The effects of factors influencing human oocyte maturation upon fertilization and preimplantation embryo development.

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A thesis submitted for the degree of Doctor of Philosophy

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April 2002

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Abbreviations

ART	Assisted reproductive technology
BMI	Body mass index
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
°C	Degrees centigrade
CG	Cortical granule
CMT	Cytoplasmic microtubule
d	day
DMAP	6-Dimethylaminopurine
EBSS	Earles balanced salt solution
EGF	Epidermal growth factor
ERK	Extracellular regulated kinase
FF-MAS	Meiosis activating sterol
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GnRHa	Gonadotrophin releasing hormone agonist
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
g	Gram
hCG	Human chorionic gonadotrophin
hr	Hour
hSA	Human serum albumin
ICSI	Intracytoplasmic sperm injection
IGF-1	Insulin-like growth factor-type 1
IU	International units
IVF	In vitro fertilization
IVM	In vitro maturation
KB	Kilobase
kDa	Kilodalton
l	Litre
LH	Luteinizing hormone
LOS	Large offspring syndrome

MAP kinase	Mitogen activated protein kinase
MIS	Meiosis-inducing substance
MPF	Maturation (or M-phase) promoting factor
MII	Metaphase II
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
mg	Milligram
ml	Millilitre
min	Minute
M	Molar
MT	Microtubule
MTOC	Microtubule organizing center
ng	Nanogram
nM	Nanomolar
OHSS	Ovarian hyperstimulation syndrome
P1	Prophase I
%	Percentage
pb	polar body
PBS	Phosphate buffered saline
PCO	Polycystic ovaries
PCOS	Polycystic ovarian syndrome
PDGF	Platelet-derived growth factor
PN	Pronuclei
PVS	Perivitelline space
RNA	Ribonucleic acid
mRNA	Messenger RNA
RT	Room temperature
TGF-α	Transforming growth factor- α
VEGF	Vascular endothelial growth factor
v/v	Volume/volume
w/v	Weight/volume

Acknowledgements

I would like to express my sincere thanks to my supervisor Geraldine Hartshorne. I am most grateful for her patience, supervision and unfailing support throughout my PhD studies and during the preparation of this thesis.

The considerable help, support & encouragement for the entirety of my PhD from all my colleagues at the CRM, particularly the lab staff are gratefully acknowledged.

Hazel Baker performed the mouse '2 cell to blastocyst' biotoxicity assay and Animal House Personnel performed the mice intraperitoneal injections of 6-12 IU hCG. Anne-Grete Byskov & Mogens Baltsen generously prepared and supplied the FF-MAS. Special thanks to Mr. Richard Kennedy and Mr. Lucas Klentzeris for performing the laparoscopic oocyte collections. My thanks are also due to Charles Tees for his help with the chromosomal and spindle analysis. From the Statistics department, I am grateful to Fotios Siannis for his advice and guidance.

I would like to express my sincere thanks and gratitude to Mr. Richard Kennedy and the R & D Committee for the financial support of these studies. For invaluable computer assistance, my personal thanks go to Mike Swann. I would also like to thank my "little sister" Sarah for her speedy and helpful retrieval of last minute references.

Above all, I am deeply indebted to my Mum and Duncan for the help, encouragement and endless support they have given me throughout the years-thank-you for believing in me.

Dedication

This thesis is dedicated to my late father, without his love, faith & support I would never have achieved the things that I feel privileged to have been able to.

You were & always will be my inspiration.

Declaration

All the results in this thesis were obtained as a result of original work by the author, under the supervision of Dr. Geraldine Hartshorne, unless otherwise stated in the text. All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree. A paper published as a result of this work is presented in Appendix V.

J.L. Cavilla

April 2002

Summary

The competence of oocytes to mature and undergo fertilization and embryonic development is known to be influenced by the conditions under which their maturation occurs. Suboptimal maturation in vitro currently limits the use of immature oocytes for embryo creation. This project examines the relationship between the conditions of in vitro maturation of human oocytes and aspects of their subsequent developmental competence through the in vitro creation and analysis of research embryos. This work is essential in defining effective and safe conditions for the use of human immature oocytes in programmes of clinical treatment to alleviate infertility.

Human immature oocytes were exposed in vitro to various concentrations of meiosis activating sterol (FF-MAS), an endogenous mediator of follicle and oocyte function, or epidermal growth factor (EGF), in the absence of other hormonal support. Their survival and further development relative to controls were measured by assessing the proportions maturing, fertilizing by sperm injection (ICSI), and cleaving in vitro. Image analysis was used to measure various dimensions of oocytes and embryos daily. A pilot study of chromosome and spindle configurations at meiotic metaphase was also conducted.

The major findings of this project are that FF-MAS supplementation of maturation medium has different positive effects upon immature oocytes arising from patient groups having different endocrine profiles and yielding differing oocyte populations. FF-MAS at 30μg/ml promotes survival of oocytes from unstimulated patients with polycystic ovaries ($p<0.025$) and promotes maturation of oocytes from gonadotrophin-stimulated patients undergoing ICSI treatment ($p<0.05$). A response to FF-MAS in terms of oocyte growth was evident in immature oocytes from both types of patients ($p<0.05$). Mature, immature and atretic oocytes from patients with PCO became significantly different in terms of oocyte diameter when cultured in FF-MAS (10 or 30μg/ml), contrasting with those cultured in control conditions, or obtained from patients undergoing ICSI treatment. However, significant ($p<0.05$) enlargement of oocyte diameter between oocyte recovery and the day of insemination occurred when oocytes from patients undergoing ICSI were cultured with FF-MAS.

The proportion of mature oocytes fertilized by ICSI was not affected by FF-MAS in the maturation culture but there was a non-significant tendency towards an improved chance of cleavage when FF-MAS was present. Embryonic development beyond early cleavage was not observed, despite conditions supportive of blastocyst production from in vivo matured oocytes, suggesting that developmental competence remained compromised even in the presence of FF-MAS. A pilot study to ascertain whether the incidence of spindle defects or chromosome irregularities in in vitro matured metaphase oocytes was influenced by the presence of FF-MAS gave equivocal results.

This project has confirmed that the ongoing development of in vitro matured oocytes may be affected by the conditions of their maturation. Specifically it has demonstrated for the first time the positive effects of FF-MAS upon human immature oocyte development in vitro, and provided the first quantitative non-invasive analysis of oocyte growth during maturation under various conditions in vitro.

Chapter 1

Introduction

"The shortage of human immature oocytes for research is the limiting factor in developing methods for using them in clinical practice" (Coskun *et al.*, 1998).

This thesis examines the maturation of human oocytes in vitro under a variety of conditions. This topic is important because little is known about human oocytes despite their widespread collection and use in infertility treatments. Oocytes are key cells, which embody the ovarian reserve of female gametes. They are laid down in early development and their quality is critical for embryo formation and the successful continuation of the human species.

In 1935, Pincus and Enzmann for the first time observed in mammals that intact oocytes, isolated from antral follicles, proceed through meiotic maturation spontaneously. In 1939, Pincus and Saunders liberated rabbit and human oocytes from their follicles in vitro and demonstrated how both matured spontaneously in less than 12 hours. This was originally considered as potentially the most appropriate way to obtain mature oocytes for in vitro fertilization (IVF) techniques (Edwards, 1965a). In retrospect, the time allowed for maturation of oocytes in vitro, based on the data of Pincus and Saunders (1939), was probably inadequate, judging from the work of Edwards (1965b).

In 1966, Edwards *et al.* made attempts to fertilize human oocytes matured in vitro. Since Steptoe and Edwards achieved the first successful human pregnancy from IVF in 1978 (Steptoe and Edwards, 1978), assisted reproductive technology has become the frontier of both infertility treatment and research (Cha and Chian, 1998). Yet, in vitro maturation (IVM) remains a largely unsuccessful approach to the treatment of infertility despite its many prospective advantages over timed collection of mature oocytes.

1.1 Oocyte development

1.1.1 Overview

1.1.1.1 Summary of follicular growth

The development of oocytes that are competent to become functional embryos is a prolonged process, starting in fetal life, when a stock of primordial follicles is formed. The oocytes are stored arrested at an immature stage of meiotic prophase, and are incompetent to develop further unless the follicle grows and provides an environment conducive to support full development. Ovulation does not occur until after puberty. In the human female, approximately seven million primordial follicles are initially formed; at birth this number has declined to 1-2 million primordial follicles, of these approximately 400 ovulate throughout the reproductive life, the rest undergoing atresia (Baker, 1963; Salha *et al.,* 1998; Hardy *et al.,* 2000). In one menstrual cycle of a mature woman, normally only a single dominant follicle achieves ovulation.

There are three successive steps in the production of a dominant follicle (Salha *et al.,* 1998)

- Growth of a daily cohort of primordial follicles.
- Recruitment of several small antral follicles by waves of pituitary gonadotrophins.
- Selection and maturation of the preovulatory follicle.

Figure 1.1 provides a diagrammatic illustration of follicular growth in the human. Although follicle growth is a continuous process occurring from early infancy until the end of the reproductive phase, follicles do not attain ovulatory sizes or produce significant quantities of oestrogen during childhood due to the absence of FSH and LH stimulation. At puberty, coordinated cyclic gonadotrophin stimulation initiates the menses and results in oocyte maturation and ovulation at midcycle (Salha *et al.,* 1998).

The mechanism that induces quiescent primordial follicles to commence growth and become follicles is not yet clear (Smitz *et al.,* 1999). Growth of a human follicle takes at least 85 days from the primary to preovulatory stage (Gougeon, 1990) and granulosa cells surrounding the oocyte are in communication with the oocyte via gap junctions throughout all stages of follicular development. The zona pellucida is

secreted by the oocyte; it separates the oocyte from the surrounding granulosa cells and is penetrated by cytoplasmic processes from the granulosa cells, which interact with the gap junctions. The gap junctions form at the points where the cytoplasmic processes contact the oocyte surface, transferring nutrients and regulatory molecules into the oocyte and also between adjacent granulosa cells, providing the basis for intercellular communication and potential syncytial coordination of the follicle (Downs, 1995).

At the primordial follicle stage, the oocyte measures approximately 30μm diameter (Gosden *et al.*, 1987). Oocyte growth and increased RNA polymerase activity is indicative of follicle growth initiation (Gosden *et al.*, 1993), and is followed by clonal and radial expansion of pregranulosa cells. At the preantral stage, the enlarged oocyte is encapsulated by >2 granulosa cell layers in close proximity without an antrum.

By the antral stage in humans, it is widely believed that the oocyte has largely completed its growth, although full maturity is not attained until the completion of follicular development and ovulation. During the antral phase of follicular growth, the oocyte is embedded in a cumulus oophorus extending on a stalk into the fluid-filled antrum, the central cavity inside the developing follicle. The antrum occupies most of the volume of a Graafian follicle just prior to ovulation, and may serve to buffer the internal environment. At antrum formation a distinction occurs between different populations of granulosa cells, the inner cumulus cells continue growing and respond to FSH by mucification, whilst the outer mural granulosa cells stop division and express P_{450} aromatase and LH receptors (Gosden *et al.* 1993).

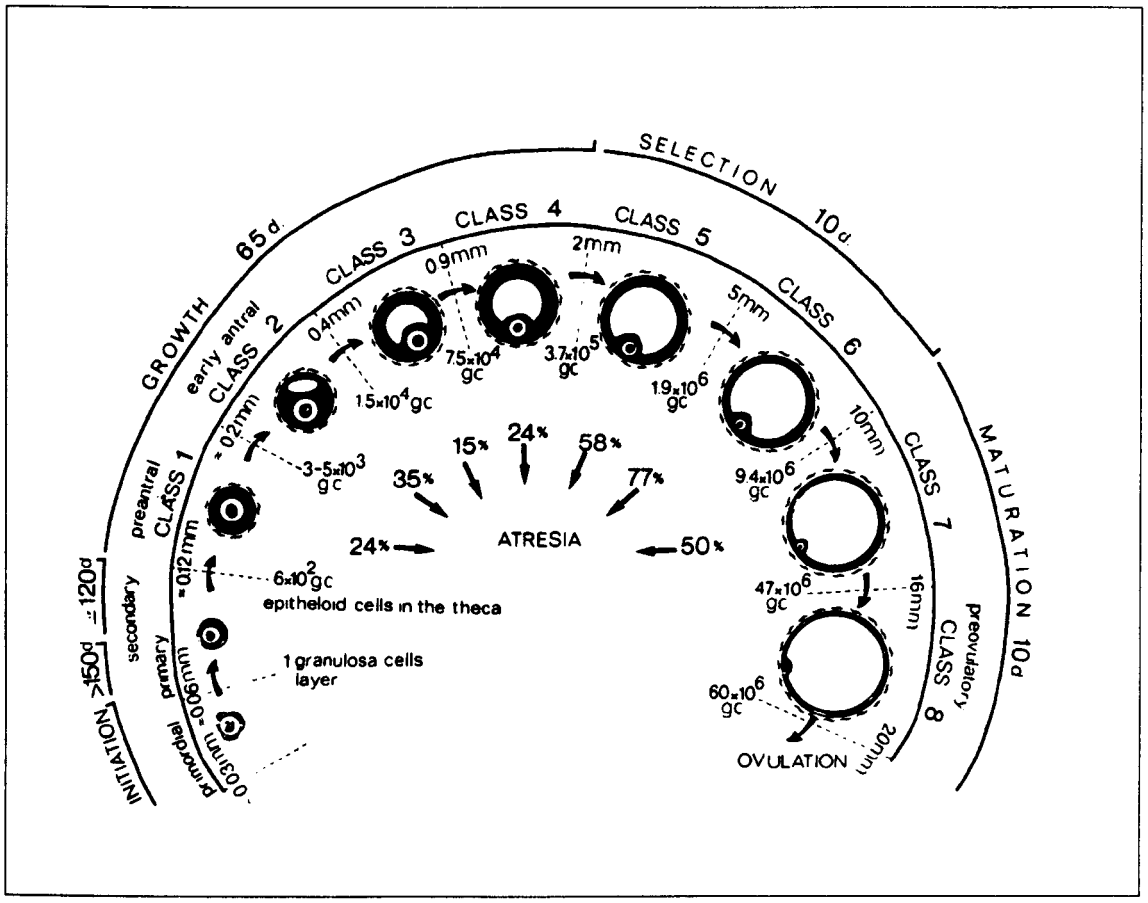


Figure 1.1 Stages of folliculogenesis in the adult human ovary and level of atresia in the eight classes of growing follicles.
The granulosa cell (gc) numbers and their corresponding estimated follicle diameter indicate the limits of each class.

(Taken from Gougeon, 1986)

1.1.1.2 Follicle selection

The follicle selected to ovulate (dominant follicle) is normally the largest healthy follicle with a diameter of 5.5-8.2mm at the start of the follicular phase (Gougeon and Lefevre, 1983). Its granulosa cells are most responsive to FSH, and modulation of FSH sensitivity may be achieved by local growth factor involvement, for example, a timely and selective activation of insulin-like growth factor-1 (IGF-1) in the chosen follicle, might promote cytodifferentation and proliferation of granulosa cells (Adashi, 1993).

The maturing follicle that achieves dominance does so via feedback inhibition of FSH secretion from the pituitary gland and increased sensitivity to FSH. The latter, combined with the fact that the dominant follicle requires less FSH to maintain preovulatory growth than is required to stimulate follicular growth at an earlier stage, enables the dominant follicle to inhibit the development of less mature follicles, without inhibiting its own growth (Salha *et al.*, 1998).

1.1.1.3 Maturation of the dominant follicle

During the follicular phase, the preovulatory follicle increases in size by both cellular multiplication and fluid accumulation in the antrum until the ovulatory LH surge. The dominant follicle begins to synthesize systemically detectable oestradiol from the mid-follicular phase. As follicle maturation occurs, the granulosa cells acquire LH receptors in response to FSH stimulation, conferring responsiveness of large follicles but not smaller ones to LH. As preovulatory follicular maturation continues, the vascularity of the follicle increases, thereby increasing the delivery of gonadotrophin to the maturing follicle as well as the systemic availability of its products, notably steroids (Zeleznik *et al.*, 1981).

The preovulatory gonadotrophin surge stimulates oocyte maturation and cumulus expansion, meiotic maturation progresses from prophase I to metaphase II (MII), reaching MII approximately 36 hr after the stimulus (Edwards, 1965b). Alterations in the oocyte cytoplasm also occur, including an increased capacity for the release of intracellular calcium (Carroll *et al.*, 1996), alteration of mRNA complement for fertilization and embryo growth prior to embryonic genome activation (Fulka *et al.*,

1998) and peripheral migration of cortical granules (Hartshorne, 1999). Cumulus expansion is a result of hyaluronic acid-rich proteoglycan matrix secretion by the cumulus cells, causing the dispersal of cumulus cells within the matrix, which are ovulated approximately 37 hr after the LH surge along with a mature MII-arrested oocyte. This expanded matrix is a critical factor for reproductive function, since it holds the oocyte in position together with its cumulus cells, facilitates follicular extrusion and oviductal fimbria capture, facilitates sperm penetration and fertilization (Salustri *et al.,* 1996; Matzuk, 2000). Potential roles in communication with the oviductal wall via sloughed cumulus cells may also be envisaged.

1.1.2 Meiosis

Meiosis is termed cell reduction; it is a form of cell division occurring only in the formation of gametes, where the number of chromosomes is reduced to a haploid number (in humans, n=23). In addition to halving the chromosome number, meiosis serves to promote genetic variation, through the mixing of maternal and paternal chromosomes and genes.

Following several mitotic divisions in the developing fetus, oogonia enter meiosis during the early second trimester of pregnancy. Meiosis is comprised of two stages: meiosis I and II, as illustrated in Figure A1 (Appendix I).

1.1.2.1 Meiosis I

Meiosis I begins in the fetal ovary and has a complex extended prophase stage, which is divided into four sub-stages (Briggs *et al.,* 1999).

Prophase I

- Leptotene- chromosomes condense, and are attached to nuclear membrane.
- Zygotene- homologous chromosomes align and homologous loci synapse. The synaptonemal complex forms, allowing sites for the exchange of genetic material (recombination). Bivalents (sets of synapsed homologues) result.
- Pachytene- shortening and thickening of bivalents, genetic recombination occurs between chromatids of paired homologous chromosomes.
- Diplotene (dictyate stage)- tetrads part, except at chiasmata, the synaptonemal complex dissolves. Oocytes remain arrested at this stage for many years prior

to ovulation or degeneration. A stable conformation of the nucleus known as a germinal vesicle is present in the oocyte.

All immature oocytes are diploid (2n) with a 4C DNA content. Progress beyond diplotene of meiotic prophase I occurs only in maturing oocytes or those in the stages of atresia where control of the oocyte by surrounding somatic cells is lost.

Metaphase I

A spindle forms at the periphery; chromosomes are randomly aligned on the equatorial plate prior to centromere repulsion and chromosome separation.

Anaphase I

The chromosomes move to opposite ends of the spindle.

Telophase I

Cell division results in the formation of daughter cells. Formation of the secondary oocyte with expulsion of first polar body (pb) (containing a set of chromosomes). The division is asymmetric due to the peripheral position of the spindle, resulting in a minimal reduction in volume of the oocyte.

1.1.2.2 Meiosis II

There is no replication of DNA or formation of a nucleus after completion of meiosis I, the cell progresses directly to MII (Veeck, 1991).

Metaphase II

A spindle reforms, the 23 chromosomes (each two chromatids) align on the equatorial plate. There is a diploid amount of DNA, but a haploid set of chromosomes, as the strands have not yet separated. The second meiotic arrest occurs and the oocyte is ovulated in this stage. Meiosis II is resumed only upon sperm penetration or parthenogenetic activation.

Anaphase II

The chromatids split at the centromeres, moving to the opposite ends of the spindle.

Telophase II

Asymmetric cell division occurs, a second pb is expelled leaving 23 maternally derived chromosomes in the oocyte together with those of the haploid sperm, to form the new, genetically unique fertilized oocyte.

1.1.3 Key stages of developmental competence

"It is only by considering all aspects of maturation that the production in vitro of mammalian eggs with a developmental potential equivalent to that of in-vivo counterparts will be achieved" (Fulka *et al.*, 1998).

Human oocyte maturation is defined as the reinitiation and completion of the first meiotic division from the germinal vesicle (GV) stage (prophase I) to MII, with accompanying cytoplasmic maturation for fertilization and early embryonic development (Cha and Chian, 1998). Although the nuclear and cytoplasmic programmes can proceed as independent processes, the acquisition of full developmental competence is conferred only when the two processes are closely integrated (Moor *et al.*, 1998; Fulka *et al.*, 1998). According to Barnes and Sirard (2000), the acquisition of developmental competence via reinitiation of meiosis is probably a common signaling or differentiation pathway that occurs in the oocyte and/or associated granulosa, regardless of whether the oocyte is destined to ovulate or degenerate.

1.1.3.1 Nuclear maturation

Nuclear maturation refers to the resumption of meiosis from prophase I arrest (P1) and the progression of meiosis to its next physiological arrest point (MII). Morphological evidence of resumption of meiosis is the disappearance of the oocyte's nucleolus and nuclear (GV) envelope, a process termed germinal vesicle breakdown (GVBD) (Eppig, 1996).

Nuclear maturation can be morphologically evaluated by observation of the extrusion of the first pb (Smitz and Cortvrindt, 1999) as illustrated in Figure 1.2.

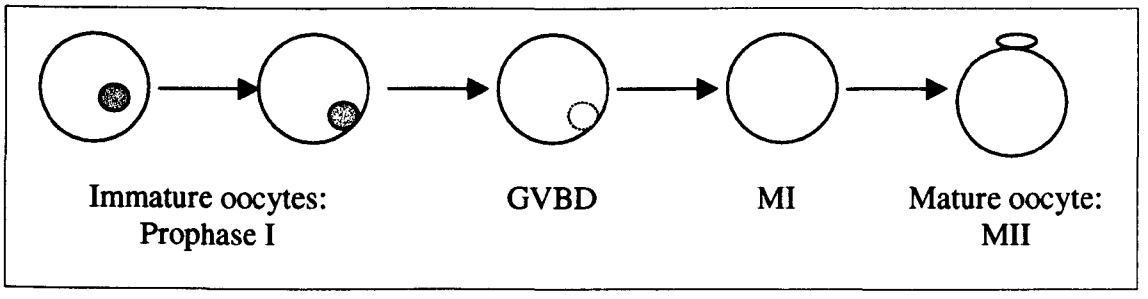


Figure 1.2 Diagrammatic illustration of the morphological features of nuclear maturation.
The GV tends to move to a peripheral location shortly prior to GVBD.

Figure 1.3 shows images of oocytes at the various stages of maturation during in vitro culture.

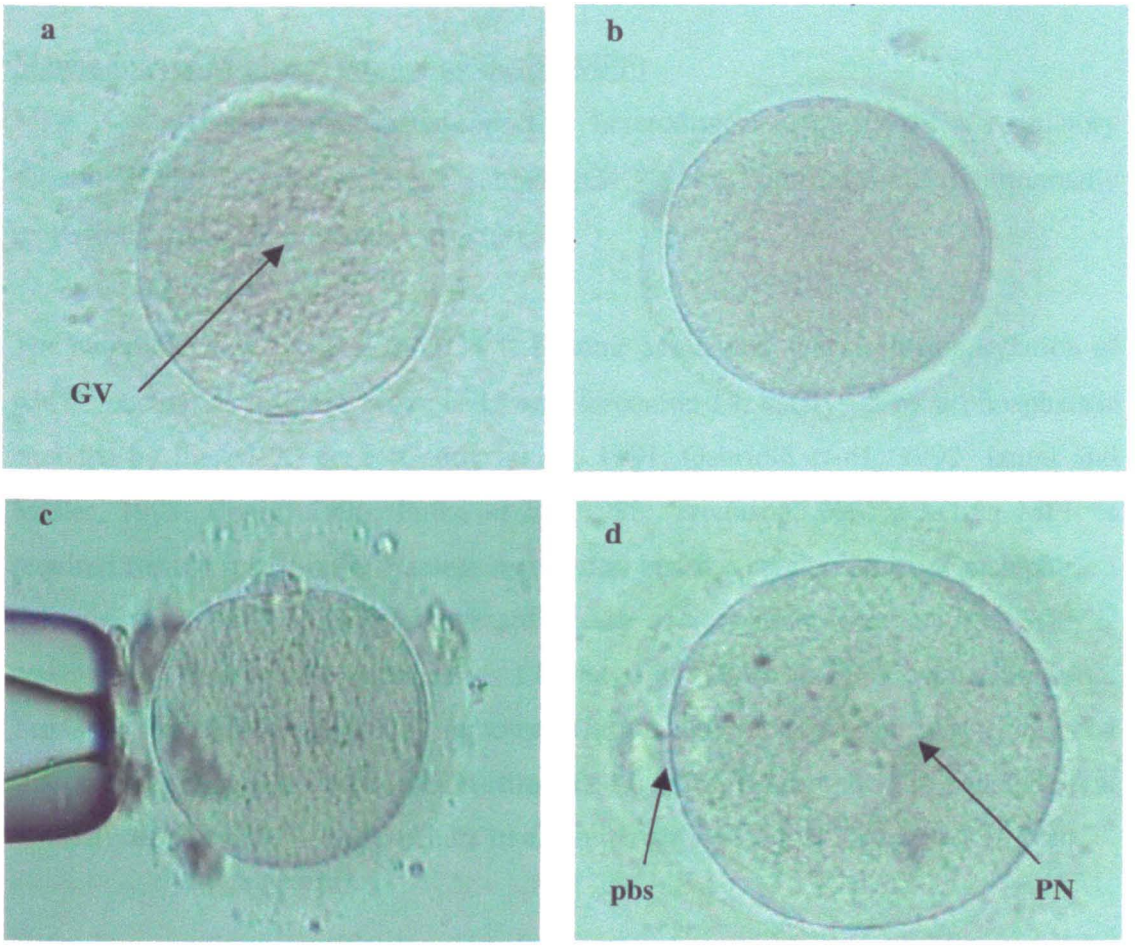


Figure 1.3 Images of the stages of human oocyte maturation in vitro. (a) Immature oocyte collected from the unstimulated ovaries of a patient with PCO. A germinal vesicle is visible in the centre of the oocyte (GV) and the oocyte has no surrounding cumulus cells (b) After one day in culture, the oocyte has undergone germinal vesicle breakdown (GVBD) (c) After two days in culture, the oocyte has one polar body and is presumed to be in MII, the oocyte is injected with a single immobilized sperm by the ICSI procedure (d) confirmation of fertilization, shown by the presence of pronuclear formation (PN) (with aligned pronucleoli) and two polar bodies (pbs).

Many of the proteins that regulate mitosis also regulate meiosis. The G_2 to M-phase transition in fully-grown oocytes is driven by maturation (or M-phase) promoting factor (MPF)- the primary molecule involved in meiotic cell cycle progression (Eppig, 1996; Fulka *et al.*, 1998; Briggs *et al.*, 1999; Trounson *et al.*, 2001).

Maturation (or M-phase) promoting factor (MPF)

MPF is a serine-threonine kinase protein heterodimer composed of a regulatory subunit, cyclin B and a catalytic subunit $p34^{cdc2}$; see Figure 1.4 for diagrammatic representation of the molecular structure.

The two molecules cyclin B and $p34^{cdc2}$ become associated, and dephosphorylation of $p34^{cdc2}$ occurs on residues tyrosine-15 and threonine-14, catalysed by a phosphatase encoded by the *cdc*25 gene (Gautier *et al.*, 1991; Gabrielli *et al.*, 1992; Izumi and Maller, 1993; Eppig, 1996; Fulka *et al.*, 1998; Trounson, 2001). Active MPF is required for the initiation of nuclear maturation and the condensation of metaphase I chromosomes, MPF activity is detected before or coincident with GVBD. MPF is activated in vivo by the endogenous LH surge at the onset of oocyte maturation. Trounson and colleagues (2001) demonstrated the activation of MPF activity in IVM studies using human oocytes from unstimulated ovaries, leading them to conclude that the activation of MPF in vitro occurs in a manner similar to that for oocytes in vivo.

Entry into anaphase I correlates with the inactivation of MPF, probably caused by proteolytic degradation of the cyclin unit of MPF (Murray *et al.*, 1989). Entry into MII requires a second increase in active MPF activity, c-mos maintains a high level of MPF, thereby arresting cell cycle progression until fertilization. Sperm penetration and the resultant increase in intracellular Ca^{2+} levels induce cyclin degradation and completion of meiosis (Fulka *et al.*, 1998; Trounson, 2001).

To summarize, oocytes acquire competence to complete nuclear maturation in at least two sequential steps (Eppig, 1996).

1. appropriate production and activation of MPF, driving entry into M-phase (GVBD and condensation of chromosomes to a prometaphase configuration).
2. inactivation of MPF, which correlates with entry into anaphase.

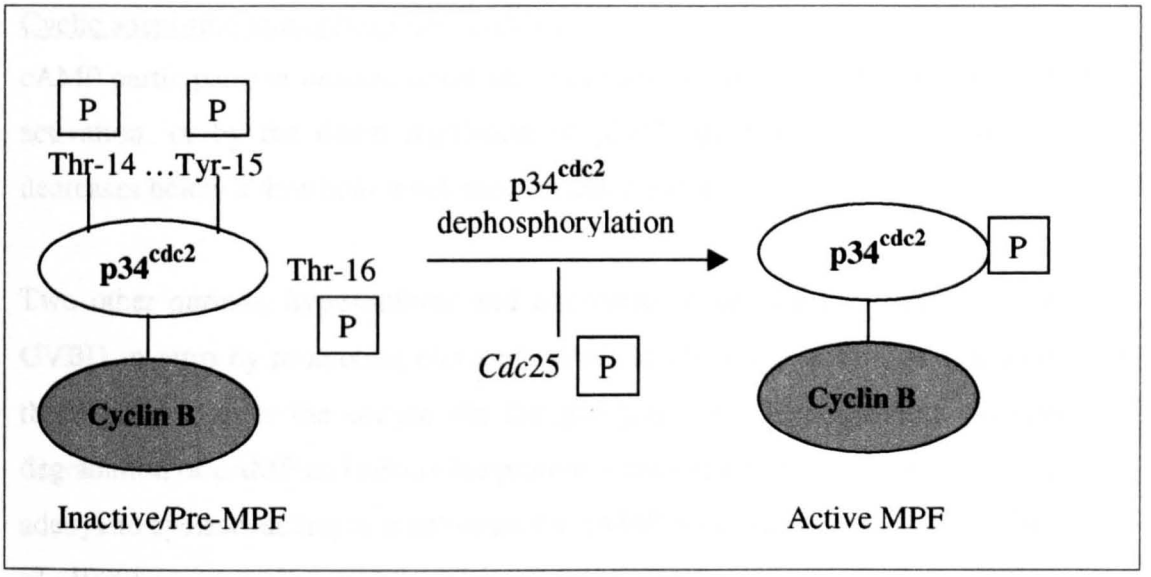


Figure 1.4. Diagrammatic illustration of the activation of maturation promoting factor (MPF).
Adapted from Eppig (1996) and Trounson *et al.* (2001).

Additional factors, which may affect meiotic progression, include MAP kinase and cAMP.

Mitogen activated protein kinase (MAP) kinase

MAP kinase is a serine-threonine kinase, alternatively known as extra-cellular regulated kinase (ERK) (Trounson, 2001); that is activated via a protein kinase cascade at the onset of oocyte maturation in mouse (Verlhac *et al.,* 1993), pig (Inoue *et al.,* 1995) and Xenopus (Haccard *et al.,* 1990). MAP kinase, although activated at the onset of oocyte maturation in mice (Trounson, 2001) is not necessarily required for GVBD in mice (Sun *et al.,* 1999b; Trounson, 2001).

In human oocytes, p42ERK2 is the main form of MAP kinase (Sun *et al.,* 1999a; Trounson, 2001). MAP kinase has not been widely studied in human oocytes, although it is known to be inactive in immature oocytes, active in mature oocytes and there is a decrease in activity after the formation of pronuclei following fertilization (Sun *et al.,* 1999a). Trounson (2001) suggests that MAP kinase may serve a similar function during human oocyte maturation to that in other mammalian species, as the pattern of activation of MAP kinase in the cell cycle is similar.

Cyclic adenosine monophosphate (cAMP)

cAMP participates in meiotic arrest and may prevent GVBD by the inhibition of MPF activation, or by the down regulation of $p34^{cdc2}$ dephosphorylation. Once cAMP decreases below a threshold level, meiosis can resume.

Two other purines, hypoxanthine and adenosine, have also been shown to prevent GVBD in vitro by promoting elevated cAMP levels in the oocyte. It is thought that these purines enter the oocyte via the gap junctions. Hypoxanthine prevents the degradation of cAMP and adenosine promotes the generation of cAMP by stimulating adenylate cyclase, acting as a substrate for cAMP production (Epigg, 1996; Briggs *et al.*, 1999).

In response to the LH surge at ovulation, gap junctions are lost following mucification of the granulosa cells, resulting from hyaluronic acid production, this causes a decrease in the levels of cAMP, below the threshold required to maintain GV status (Briggs *et al.*, 1999).

1.1.3.2 Cytoplasmic maturation

Cytoplasmic maturation refers to the processes that prepare the oocyte for fertilization, activation, formation of PN and preimplantation development. Similarly to nuclear maturation, competence to undergo cytoplasmic maturation is acquired in sequential steps by GV-stage oocytes. Although nuclear and cytoplasmic maturation occur in synchrony, the acquisition by GV-stage oocytes of competence to undergo cytoplasmic maturation is independent of competence to complete nuclear maturation. Most deficiencies in oocytes during maturation are believed to be associated with cytoplasmic reprogramming rather than with meiotic progression; and the effects of cytoplasmic aberrations are normally associated with cleavage, blastulation and the peri-implantation stages, rather than early development (Moor *et al.*, 1998).

Protein synthesis

Proteins are synthesized in growing oocytes from both nuclear and mitochondrial transcripts for use by the oocyte, for communication with surrounding cells and for early embryo development (Briggs *et al.*, 1999). Oocyte mRNA that will be translated

during oocyte maturation is stored in a stable, dormant form until after GVBD (Bachvarova, 1985). Moor *et al.* (1998) suggested that the reduced developmental potential in human oocytes matured in vitro might be attributable to abnormal cytoplasmic maturation, as well as sub-optimal culture conditions and incomplete growth. Trounson (2001) studied the protein content of in vitro matured MII human oocytes, obtained from unstimulated ovaries. A reduced protein content was observed compared to in vivo matured MII oocytes retrieved from stimulated ovaries. The proteins not detected in oocytes matured in vitro probably include molecules essential for cell cycle regulation and normal embryo development.

Inositol lipids and calcium

Sperm binding and fusion promote Ca^{2+}-dependent changes that result in oocyte activation. Ca^{2+} is released from the intracellular stores via an IP_3-dependent signal transduction mechanism (Homa, 1995). The oocyte's capacity to release intracellular Ca^{2+} is relatively low during the early stages of maturation, but reaches maximum sensitivity at MII (Fujiwara *et al.*, 1993). The release of Ca^{2+}, essential for oocyte activation and pronuclear formation, normally coincides with nuclear maturation (Eppig, 1996).

Glutathione production

The levels of glutathione in oocytes rise during maturation (Perreault, 1988). Glutathione is a reducing agent, which aids sperm decondensation and may play a role in the formation of the male pronucleus, as well as potentially protecting from pathological oxidation processes and free radicals (Yoshida *et al.*, 1993; De Matos *et al.*, 1996).

Competence for the release of cortical granules

Cortical granules control the permeability of the zona pellucida to sperm, occupying a peripheral position below the oolemma. The fusion or penetration of a single spermatozoon into the oocyte triggers cortical granule release, blocking polyspermy. The exocytosis of cortical granules in response to Ca^{2+} ionophore increases with the progression of maturation (Sathananthan and Trounson, 1982; Ducibella, 1996).

Follicle size

The size of the follicle from which the oocyte originates influences its developmental capacity, as aspects of developmental competence are acquired during follicular growth (Tsuji *et al.*, 1985).

A significantly higher percentage of human oocytes retrieved during the follicular phase of the menstrual cycle from follicles of 9-15mm diameter underwent GVBD, than did oocytes collected during the luteal phase from follicles of 3-4mm in diameter (60% and 48% respectively). The percentage of oocytes reaching the MII stage did not differ significantly between the two groups (Whitacre *et al.*, 1998). Conversely, a minimum follicle diameter of 5mm was reported by Wynn *et al.* (1998) to be required for oocytes capable of in vitro maturation.

It is possible that follicles of <10mm in diameter contain developmentally less competent oocytes than larger follicles (Dubey, 1995; Trounson *et al.*, 1998). The ability of gonadotrophin priming to influence developmental competence is dependent on the stage and size of follicle development (Barnes, 2000).

Oocyte size

The human oocyte has a size-dependent ability to resume meiosis and complete maturation, with an oocyte diameter of >115μm reported to be necessary for the progression from GVBD to MII (Durinzi *et al.*, 1995).

A better understanding of the minimal requirements for oocyte growth and maturation will enable the development of consistent techniques of in vitro culture of follicles and in vitro maturation of oocytes. In vitro maturation of human oocytes is currently undertaken with limited success, due to our incomplete knowledge of the factors controlling developmental competence.

A better understanding of the development of the oocyte is crucial, because the quality of embryos depends on the maternal endowment of RNA and protein molecules laid down during oogenesis, their stability and controlled expression. Molecular markers of oocyte viability are needed to inform the current morphological

assessments of oocyte/embryo quality, which do not provide a reliable indication of the prospect of pregnancy (Briggs *et al.*, 1999). With the continuation of ongoing research into these areas worldwide, the resulting knowledge gained and consequently increased pregnancy rates; IVM is expected to become a clinically viable technique for infertility treatment.

1.2 In vitro growth of follicles

Methods for the long-term culture of whole human follicles are at present in their infancy; therefore oocytes retrieved from enlarged antral follicles are usually used in IVM studies. However, in future, it is likely that the technology for early follicle growth in vitro will be combined with that for in vitro maturation of oocytes to extend the period of oocyte culture. Hence a brief description of follicle techniques is provided here.

Prospective applications for oocytes obtained from follicular culture include oocyte donation, oocyte storage, IVF, animal production technology, support of endangered species and in the experimental study of follicular and oocyte development (Gosden *et al.*, 1993; Salha *et al.*, 1998; Cortvrindt and Smitz, 2001).

As yet, no systems permit complete in vitro culture of early follicle stages in large animals or humans, although parts of folliculogenesis have been successfully reproduced in vitro (Cortvrindt and Smitz, 2001). To date, in vitro culture from primordial follicle to live birth has been achieved only in mice (Eppig and O'Brien, 1996). However, less than 2% of fertilized oocytes developed to blastocyst stage. Of 190 embryo transfers, only one live birth resulted, which developed severe obesity, liver problems and neurological damage nine months later (Eppig and O'Brien, 1998). The live birth was achieved following a two-step strategy; first the ovaries (containing only newly formed primordial follicles) of newborn mice were grown in organ culture for a period of eight days to allow the development of growing oocytes enclosed by 1-3 layers of granulosa cells. A normal number of primordial follicles initiated growth and developed to the secondary follicle stage by day 8, similar to development after 8 days in vivo. This was followed by isolation of the developing oocyte-granulosa cell

complexes, which were cultured for a further 14 days to complete oocyte development.

The total development period of ~three weeks corresponds to the estimated time required for full growth in vivo (Pedersen, 1970). According to Gougeon (1986), a period in excess of six months is required for the equivalent in vivo with human primordial follicles, due to the differing growth rates between small and large mammals, however it is possible that artificial manipulation in vitro might enable the schedule to be hastened.

Murine follicles are ideal for developing a culture system, since they are small, readily available and grow to full size within a short time-span. The formation of primordial follicles, which have an oocyte in first prophase of meiosis and a single layer of flattened granulosa cells, occurs synchronously within a few days after birth in rats and mice (Fortune *et al.*, 2000). Activation of a few primordial follicles begins almost immediately thereafter, yielding the first group of antral follicles around day 15 postpartum (Hirshfield, 1991). Thus, the timing of follicle formation and the initiation of primordial follicle activation in rodents, coupled with an ovarian size that allows organ culture of whole ovaries, make them very useful models.

Interspecies differences make it difficult to transpose techniques between species, there are various problems associated with adapting such a system for use with large species. In contrast to rodents, follicle formation in ruminants and primates begins during fetal development and occurs over a long period of time, so that some follicles are activated and leave the resting pool before others have been formed (Henricson and Rajakoski, 1959; Russe, 1983). These developmental processes are thus protracted and asynchronous in these larger species, making them more difficult to study (Fortune *et al.*, 2000). The follicles of primates and ruminants have a much thicker theca than in rodents, which restricts oxygen and nutrient transport to the centre of the follicle, which tends to become necrotic. Removal of the theca externa may overcome this problem, as this layer may not be essential for follicle development (Gosden *et al.*, 1993). Culture systems using small pieces of ovarian cortex obtained from fetal ovaries are used for domestic species and primates, as the

larger size of ovaries precludes the whole-ovary culture system used with rodents. There are also interspecies differences in regulation and timing of maturation; mouse oocytes acquire GVBD competence at an earlier stage of follicular development than MII competence, which is attained only when the oocyte has reached maximum size soon after follicular antrum formation (Erickson and Sorensen, 1974; Sorensen and Wassarman, 1976). In contrast, at antrum formation, ungulate oocytes (bovine and porcine) have not reached maximum size and exhibit low meiotic competence (McGaughey *et al.*, 1979; Motlik *et al.*, 1984; Motlik and Fulka, 1986). Even after the acquisition of maximum oocyte size during antral growth, MII competence is low and increases only with increasing follicular size (Gilchrist *et al.*, 1995).

Despite difficulties encountered with for example, the duration of the follicular growth span from the primordial to Graafian stage, changes in the nutritional requirements of the cells, cellular interactions, morphogenesis and the sheer increase in volume associated with antrum formation, human follicle culture is making progress. Picton and Gosden (2000) suggest that at present, the best strategy is likely to be initiation of follicle growth in situ in slices of ovarian cortex, isolation of the follicles or granulosa-oocyte complexes once they have progressed to preantral stages for individual culture. Followed by IVM of the oocytes within their cumulus cells once full oocyte growth has been achieved. The preservation of cellular interactions and the phenotype of follicle cells is likely to increase the chances of succeeding at each stage, as these provide the physiological environment in which oocytes develop. Culture of intact follicles beyond ~500-1000μm in diameter is unlikely to succeed due to failure of oxygen diffusion (Gosden *et al.*, 1986). Therefore, beyond this point, oocyte isolation or a non-intact follicle culture system, as in mice is likely to be essential (Cortvrindt *et al.*, 1996).

At present, development of follicles in vivo has produced more immediate prospects for clinical application and in animals can restore fertility after re-aggregation and grafting (Carroll and Gosden, 1993). Experimentation in sheep, whose ovaries resemble those of humans in size and follicle and fibre composition, and in humans, has demonstrated the successful autografting of primordial follicles in cortical tissue pieces (Gosden *et al.*, 1994; Nugent *et al.*, 1997). Growth and maturation of

cryopreserved human primordial and primary follicles can be achieved by homologous transplantation of the frozen-thawed ovarian tissue (Nugent *et al.*, 1997; Smitz and Cortvrindt, 1999), although there was variable survival of the follicles within the transplants. No human pregnancies have resulted from these auto transplants to date

The technique of in vitro growth of immature follicles in combination with in vitro maturation and cryopreservation, promises to be a powerful technology in assisted reproduction. However, over the past decade there has been no real progress in applying these techniques for use in animals and humans with follicles that undergo a long growth period (Telfer *et al.*, 2000). Although it is possible to grow primordial follicles to pre-antral stages in slices of ovarian tissue, and support antrum formation in isolated pre-antral follicles, we are still some considerable way from growing and maturing pre-antral follicles to the pre-ovulatory stage in vitro (Hardy *et al.*, 2000). However, Gosden (2000) predicts that eventually it will be possible to produce viable embryos from oocytes that have been grown and matured entirely in vitro. This achievement will provide the foundations of a technology that not only has important practical application but also provides the most valuable model for investigating oogenesis.

1.3 The current state of IVM of human oocytes

1.3.1 Background

The final 36 hr of human oocyte formation are critical for the normal functioning of the resulting gamete. During this time, oocytes resume meiosis from their prenatal arrest in diplotene of meiotic prophase I, and progress to metaphase II. They also undergo cytoplasmic maturation (section 1.1.3.2) in preparation for fertilization and early embryo development, which is largely dependent upon oocyte constituents until after embryonic genome activation, which occurs principally between the 4-8 cell stages of embryonic growth (Braude, 1988).

It has been known for many years that immature human oocytes removed from large follicles will mature spontaneously (Pincus and Enzmann, 1935; Edwards, 1965a,b); however, the developmental competence of in vitro matured human oocytes is low

(Veeck *et al.*, 1983; Barnes *et al.*, 1996; Coskun *et al.*, 1998), probably due to disruption of the normal follicular control mechanisms regulating this important stage of development. When oocytes are aspirated from antral follicles early in the follicular phase, follicular and oocyte growth are incomplete at the time of oocyte retrieval, and some follicles may already have initiated the process of atresia. Collected oocytes are usually matured in the 48 hr subsequent to retrieval, a shortened period of time compared with the natural cycle, in which nuclear maturation follows the luteinization signal and cytoplasmic maturation is a progressive phenomenon (Cheung *et al.*, 2000; BFS Policy and Practice Sub-Committee, 2001). It is possible that the maturational and developmental anomalies observed in in vitro matured oocytes are attributable to their truncated growth phase and thus the inability to complete all the necessary transcriptional and translational changes required for complete maturation and developmental competence.

Following the work of Lonergan *et al.* (1997), Anderiesz *et al.* (2000) artificially extended the pre-maturation growth phase of human and mice oocytes in vitro using 6-Dimethylaminopurine (DMAP) which reversibly inhibits GVBD. DMAP blocks GVBD and cell cycle progression in immature oocytes by inhibiting the post-translational dephosphorylation of $p34^{cdc2}$ that triggers MPF activity (Jessus *et al.*, 1991), but does not interfere with protein synthesis (Rime *et al.*, 1989; Fulka *et al.*, 1991; Trounson *et al.*, 2001). It was hypothesized that DMAP treatment may synchronize nuclear maturational events in oocytes, but there was no evidence of this effect in either mouse or human oocytes. DMAP treatment of human oocytes had no effect on fertilization or development to the blastocyst stage, but increased the developmental capacity of mouse embryos. Anderiesz *et al.* (2000) concluded that lengthening the prematuration growth phase, by temporarily inhibiting kinase activity with DMAP, does not directly improve oocyte developmental competence.

Due to the low developmental competence of in vitro matured oocytes, in vivo maturation has been preferred for oocyte preparation for IVF despite the need for large doses of exogenous hormones, with their attendant risks and costs (Russell, 1999). This is in contrast to some animal species where IVM is commonly used to obtain viable oocytes for research or commercial purposes (Trounson *et al.*, 1996), yet

this is not without problems, as a proportion of the animals thus produced are abnormal (Young *et al.*, 1998; Sinclair *et al.*, 2000).

The challenge with human oocytes is to provide optimal conditions for IVM by mimicking the microendocrine environment of the developing follicle to enable the immature oocyte to achieve nuclear and cytoplasmic maturation. When utilising in vitro matured oocytes, conditions for fertilization and culture as well as uterine receptivity must also be optimal if implantation is to occur (Russell, 1998). IVM is particularly challenging in the human because folliculogenesis is a lengthy process encompassing many complex changes in the oocyte and its surrounding follicle cells (Hardy *et al.*, 2000) which are known to have a bearing upon later developmental competence. Moreover, disruption of these processes may potentially have devastating effects upon development.

The production of embryos in vitro exposes them to hazards not normally encountered in vivo, and as a result, there have been unforeseen consequences including the large offspring syndrome (LOS), also termed the fetal oversize syndrome (Walker *et al.*, 1992; Behboodi *et al.*, 1995; Farin and Farin, 1995; Walker *et al.*, 1996; Thompson, 1997; Young *et al.*, 1998; McEvoy *et al.*, 2000) which has not however been evident in human IVF offspring. The birth of grossly enlarged offspring was the first marked and unexpected adverse consequence of culturing cattle or sheep zygotes ex utero (McEvoy *et al.*, 2000). However, it is also associated with an increased abortion rate, increased gestation lengths, physical abnormalities and increased mortality and morbidity (Maxfield *et al.*, 1998; Ranilla *et al.*, 1998; Sinclair *et al.*, 1999; Telfer *et al.*, 2000). Altered genomic imprinting is a likely cause of this syndrome (Sinclair *et al.*, 2000); imprinted genes (i.e. genes which express only the maternal or paternal allele) play key roles in the control of fetal growth (Khosla *et al.*, 2001) and are possible candidates for involvement in livestock LOS (Young and Fairburn, 2000).

Although there is no published evidence for a human equivalent to the LOS as a consequence of embryo production in vitro, it is known that oocytes, zygotes and embryos up to and beyond blastocyst stages of development are extremely sensitive to their environment, whether natural or artificial, and their normal development can be

threatened in many ways. Experience from the culture of cattle and sheep embryos to the blastocyst stage of development may help to avoid some of these dangers (McEvoy *et al.*, 2000).

1.3.2 Content of media used for IVM

The content of the medium used for IVM may affect the outcome. A key experiment in the 1990s, which re-awakened interest in human IVM, used human follicular fluid to supplement the culture medium (Cha *et al.*, 1991) but, with the growing preference for defined media, the roles of individual factors are now being assessed and the inclusion of biological fluids is declining.

Various hormones included in the culture medium have promoted oocyte maturation and subsequent embryo development, for example, epidermal growth factor (EGF, Gómez *et al.*, 1993a,b; Goud *et al.*, 1998) or follicle stimulating hormone (FSH, Barnes *et al.*, 1996; Durinzi *et al.*, 1997) with or without human chorionic gonadotrophin (hCG, Jaroudi *et al.*, 1997; Cha *et al.*, 2000; Liu *et al.*, 1997).

Granulosa cells contain mRNA for FSH receptors from very early in follicle development (Oktay *et al.*, 1997), whereas LH receptors are induced much later, in response to FSH stimulation, and are principally located at the periphery of large follicles (Amsterdam *et al.*, 1975). Receptors for the gonadotrophins are lacking on oocytes, so any effects observed must be mediated via the attached cumulus cells (Hartshorne, 1999). Cumulus cells are highly metabolically active and their steroid production may be influenced by hormonal supplementation in vitro (Durinzi *et al.*, 1997).

1.3.3 Intracytoplasmic sperm injection (ICSI) in human IVM programmes

ICSI is the injection of a single spermatozoon into the cytoplasm of the oocyte. There is evidence that removing human oocytes from their normal follicular environment before luteinization yields oocytes with compromised ability to be fertilized, as exemplified by the failure to elicit normal calcium signalling in response to sperm-oocyte fusion (Herbert *et al.*, 1997). Consequently, intracytoplasmic sperm injection (ICSI) has been employed almost universally to achieve fertilization in human IVM

programmes (BFS Policy and Practice Sub-Committee, 2001). ICSI of in vitro matured oocytes has increased the likelihood of normal fertilization; overcoming problems with zona hardening due to extended culture (Barnes *et al.*, 1995, 1996; Nagy *et al.*, 1996; Cha and Chian, 1998; Hwang, *et al.*, 2000), and failure of the cortical reaction probably due to the inadequate peripheral migration of cortical granules during maturation.

1.3.4 Clinical pregnancies resulting from IVM

The full developmental competence of an oocyte and an embryo can ultimately be evaluated only by live births. In 1983, Veeck *et al.* reported the first live birth resulting from successful IVM as part of an IVF programme in which patients received ovarian stimulation drugs. Subsequently, Cha *et al.* (1991) reported IVM with unstimulated oocytes in a donor oocyte programme.

Following this there have been a number of reports of the successful use of IVM resulting in clinical pregnancies (Veeck *et al.*, 1983; Trounson *et al.*, 1994; Barnes *et al.*, 1995; Nagy *et al.*, 1996; Edirisinghe *et al.*, 1997; Jaroudi *et al.*, 1997; Liu *et al.*, 1997; Cha and Chian, 1998; Russell *et al.*, 1998; Thornton *et al.*, 1998; Tucker *et al.*, 1998; De Vos *et al.*, 1999; Chian *et al.*, 1999a,b; Jaroudi *et al.*, 1999; Mikkelsen *et al.*, 1999; Cha *et al.*, 2000; Chian *et al.*, 2000; Mikkelsen *et al.*, 2000; Smith *et al.*, 2000; Abdul-Jalil *et al.*, 2001;Chian *et al.*, 2001; Wu *et al.*, 2001) however the live birth rate associated with IVM remains low. Pregnancy rates of approximately 2% result from the transfer of up to three embryos, derived from oocytes matured in vitro. The simultaneous transfer of many more embryos derived from oocytes matured in vitro may result in higher pregnancy rates (Cha *et al.*, 2000).

1.3.5 Role of prior ovarian stimulation in IVM

To achieve ovarian stimulation for IVF, gonadotrophin-releasing hormone (GnRH) agonists or antagonists are used to suppress the secretion and release of endogenous gonadotrophins from the pituitary gland. Exogenous FSH is administered to induce multiple follicular growth and the final phase of oocyte maturation is induced by a dose of hCG, to emulate the LH surge.

Various protocols including partial or minimal exogenous hormonal stimulation are used in preparation for IVM of human oocytes. In preliminary studies of the treatment of women for one or three days with recombinant human FSH (rFSH) early in the follicular phase, no difference in the recovery rate of oocytes, maturation, fertilization or development in culture was demonstrated (Trounson *et al.*, 1998). Mikkelsen *et al.* (1999) confirmed this finding. After the treatment of women with rFSH for three days, on days 3-5 of the cycle, no benefit was observed from extending the rFSH pre-treatment from three to six days to produce follicles >10mm in diameter.

However, Wynn *et al.* (1998) administered a truncated course of 600IU rFSH over five days (300 IU on day two, 150IU on day four and six). A mean of 7.5 oocytes was recovered after rFSH treatment compared with 5.2 oocytes from untreated oocytes. After 48 hr in culture, supplemented with FSH and hCG, significantly more oocytes completed maturation to MII following FSH stimulation than in untreated women (71.1% and 43.5% respectively). Significantly fewer degenerating oocytes were seen both at the time of collection and after 48 hr of culture from patients treated with rFSH.

Suikkari *et al.* (2000) also investigated the use of minimal ovarian stimulation of recruitable follicles, in order to produce competent oocytes for IVM and fertilization. In a natural menstrual cycle, serum FSH begins to increase in the late luteal phase, therefore, low dose rFSH was administered starting in the late luteal phase in two groups of women, those with regular menstrual cycles and women with PCO and irregular cycles. In women with anovulatory cycles, a withdrawal bleed was induced by using 600mg daily vaginal micronized progesterone for ten days. Low-dose rFSH was commenced on the ninth day of progesterone administration. Oocytes were retrieved after withdrawing rFSH for two-five days. It was postulated that low dose rFSH priming of follicles would support the growth of multiple follicles, thereby increasing the number of immature oocytes retrieved for IVM. A good yield of immature oocytes (11.2 and 11.5: regular and irregular menstrual cycles, respectively) was retrieved in both groups of women, 71% of viable oocytes reached MII after 44 h in culture and 64% of these fertilized after ICSI. The oocyte maturation and fertilization rates did not differ significantly between the two groups of women.

Trounson *et al.* (2001) suggest that the failure to achieve a substantial improvement in the number and developmental competence of oocytes by pre-treatment with FSH indicates that maturation in vitro is not limited by the growth phase of follicles in the ovaries. Furthermore, there would be no clinical application of FSH pre-treatment prior to immature oocyte recovery, unless a very substantial benefit could be demonstrated for maturation and development to term.

The time course of oocyte GVBD and maturation is different between GV oocytes retrieved from stimulated and unstimulated ovaries. Data from Cha and Chian (1998) demonstrated that although the final percentages of GVBD (after 27 hr of culture) for oocytes derived from stimulated and unstimulated ovaries did not differ (83.3% and 87.5% respectively), GVBD occurred significantly earlier in oocytes retrieved from stimulated ovaries. After 12 h of culture, 80% of GV oocytes from stimulated ovaries had undergone GVBD, whereas there was no change in GV oocytes from unstimulated ovaries at the same time point. As a result, the completion times for nuclear maturation, shown by extrusion of the first pb differed, ~75% of oocytes from stimulated ovaries reached MII by 30 hr of culture whereas 75% of oocytes from unstimulated ovaries reached MII by 42-45 hr (Cha and Chian, 1998; Trounson *et al.*, 2001). The final proportions of maturing oocytes derived from stimulated and unstimulated ovaries were not different (75% and 77.5% respectively). The exposure of the follicles to FSH and hCG in stimulated ovaries prior to oocyte retrieval may account for the difference in timing of GVBD.

Gonadotrophin therapy leads to the growth of multiple follicles, this is reflected in a rise of oestradiol concentrations, and therefore oocytes retrieved from stimulated ovaries have been subject to a predominantly oestrogenic milieu (Cobo *et al.*, 1999). Furthermore, it has been shown that the addition of oestradiol to oocyte maturation medium can directly influence the quality of the maturing oocyte. Whilst no apparent effect of oestradiol on either GVBD or further progression of meiosis was observed, increased fertilization and cleavage rates were seen after IVM. Oestradiol induces a series of transient increases in the intracellular free Ca^{2+} concentration, which contributes to the oocyte's capacity for fertilization and early post-fertilization development (Tesarik and Mendoza, 1995).

IVM and fertilization have been reported for human oocytes retrieved from cycles after the administration of exogenous hCG in vivo (Veeck *et al.*, 1983; Prins *et al.*, 1987; Dandekar *et al.*, 1991; Toth *et al.*, 1994; Janssenswillen *et al.*, 1995; Nagy *et al.*, 1996; Edirisinghe *et al.*, 1997; Farhi *et al.*, 1997; Goud *et al.*, 1998; Thornton *et al.*, 1998; Tucker *et al.*, 1998; De Vos *et al.*, 1999; Chian *et al.*, 1999a,b; Chian *et al.*, 2000; Cavilla *et al.*, 2001; Abdul-Jalil *et al.*, 2001).

The above studies differ in many aspects, e.g. number of immature oocytes for in vitro maturation, time in culture, culture medium used and maturation, fertilization and embryo cleavage rates. However, all studies that replaced embryos resulting from IVM reported subsequent clinical pregnancies (Veeck *et al.*, 1983; Nagy *et al.*, 1996; Edirisinghe *et al.*, 1997; Thornton *et al.*, 1998; Tucker *et al.*, 1998; De Vos *et al.*, 1999; Chian *et al.*, 1999a,b; Chian *et al.*, 2000; Abdul-Jalil *et al.*, 2001).

Immature oocytes retrieved after the in vivo administration of exogenous hCG can be matured in culture without gonadotrophin or steroid supplement (Veeck *et al.*, 1983; Dandekar *et al.*, 1991) but the culture medium usually contains the patient's own serum and occasionally granulosa cells recovered from the same follicle or from follicles that contained a mature follicle.

Four of the IVM studies involved cryopreservation (Toth *et al.*, 1994; Edirisinghe *et al.*, 1997; Thornton *et al.*, 1998 and Tucker *et al.*, 1998) three of which resulted in clinical pregnancies. Results from Toth *et al.* (1994) demonstrated that prophase I oocytes from stimulated IVF cycles are able to survive cryopreservation and resume meiosis to achieve full nuclear maturation post-thaw. In addition, cryopreserved oocytes retained the same capacity for fertilization and development as control (cultured, non-cryopreserved immature) oocytes. Edirisinghe *et al.* (1997) reported a case resulting in a live birth following the transfer of cryopreserved embryos generated from ICSI carried out on the second day after oocyte recovery of three in-vitro matured MI and two GV stage oocytes. All five zygotes were cryopreserved at the pronuclear stage, then thawed and cultured for 24 hr prior to the transfer of two embryos in a cycle stimulated with low doses of follicle stimulating hormone.

In a variation of natural cycle IVF, Thornton *et al.* (1998) recovered immature GV oocytes from follicles secondary to the one dominant follicle after administration of in vivo hCG to trigger ovulation. These follicles were ≤ 12mm, oocytes were matured in standard culture medium or Hams F10 + 50% follicular fluid as described by Cha *et al.* (1991). No significant differences were observed in maturation or fertilization rates. Two births resulted, one of the births resulted from one of six transfers of cryopreserved embryos derived from oocytes matured in vitro. Chian *et al.* (1999a,b, 2000) adopted a similar approach for PCOS patients with irregular menstrual cycles, supplementation of the maturation medium with gonadotrophins and serum additives yielded good pregnancy rates.

In 1998, Tucker *et al.* reported a pregnancy resulting from the cryopreservation of GV stage oocytes, followed by successful thawing, IVM and ICSI. A total of 29 oocytes were cryopreserved, 16 at MII and 13 at the GV stage. No MII oocytes survived the thaw procedure, however, three GV oocytes survived, after 30 hr IVM, two oocytes had matured, which fertilized after ICSI. Tucker *et al.* (1998) concluded that this study proves the feasibility if not the efficiency of using immature oocytes for cryostorage, coupling both cryopreservation and IVM.

In cases of recurrent ovarian hyperstimulation syndrome (OHSS) (or for those at high risk of developing OHSS) with clinical IVF, immature oocytes can be retrieved after gonadotrophin treatment, but without hCG administration. Jaroudi *et al.* (1997) reported such a case.

1.3.6 Different stimulation

For patients wishing to avoid large doses of FSH, or for patients for whom such superovulation is unaffordable, retrieval of oocytes from small non-dominant follicles in natural ovulatory cycles, after minimal ovarian stimulation with clomiphene citrate (Thornton *et al.*, 1998; Trounson *et al.*, 2001) and subsequent IVM offers an alternative option and a chance of achieving pregnancy.

1.3.7 Importance of PCO

In 1994, Trounson *et al.* developed methods for the recovery of oocytes from the ovaries of patients with PCO, in which the dominance of a particular follicle fails to occur and the cohort of growing follicles accumulates in the cortex. These follicles range in diameter from about 3-8mm and remain under an androgen-dominated environment due to increased thecal cell secretion of androgens and a blockage of aromatization in the granulosa cell compartments (Almahbobi and Trounson, 1996; Trounson *et al.*, 2001) (Section 1.4).

In 1996, Barnes *et al.* demonstrated significantly lower maturation, fertilization and embryo development rates with oocytes recovered from women with PCOS, compared with those recovered from regularly cycling women without PCOS. They suggested that the abnormal endocrine environment and stasis of follicular growth in PCOS patients may disrupt the oocytes, although incipient follicular atresia does not appear to reduce the developmental competence of human oocytes (Barnes *et al.*, 1996). However, blastocysts have been produced and live births have occurred as a result of IVM of oocytes from women with PCOS (Trounson *et al.*, 1994; Barnes *et al.*, 1995; Cha and Chian, 1998; Chian *et al.*, 1999a,b, 2001). Furthermore, the higher oocyte yield from the increased numbers of antral follicles in such patients may potentially compensate for lower individual viability of oocytes (Trounson *et al.*, 1996).

Recent reports of live births following IVM treatment of women during unstimulated cycles (Cha *et al.*, 2000; Chian *et al.*, 2000; Chian *et al.*, 2001) have emphasized the significant potential of IVM treatment, especially for women with polycystic ovaries (Abdul-Jalil *et al.*, 2001). IVM can be used to achieve pregnancy for women with PCOS and irregular menstrual cycles, after a withdrawal bleed, induced with progesterone, hCG 10-14 days later, followed by oocyte recovery 36 h later (Chian *et al.*, 1999a,b, 2000).

When performing an immature oocyte recovery in a patient with PCO and irregular menstrual cycles, it is not known whether the follicles are atretic. Inducing a withdrawal bleed causes the endometrium to develop afresh, and feeds back to the

pituitary to reduce the levels of FSH. Upon withdrawal of the steroids used to induce the bleed, the negative feedback to the pituitary is reduced. Therefore, the FSH level starts to rise and a new cohort of antral follicles commence growth. The retrieval of these oocytes at late stage of the follicular phase before the LH surge enables oocytes to be retrieved from follicles less likely to be atretic, than from patients with PCO who had not had a withdrawal bleed.

In 1999(a,b), Chian *et al.* reported a clinical pregnancy rate of 39% per cycle after hCG priming before immature oocyte retrieval and IVM in women diagnosed with polycystic ovaries. The patients had irregular menstrual cycles and withdrawal bleeding was induced by the administration of intravaginal progesterone for 10 days, hCG was administered 10-14 days after steroid withdrawal. Transvaginal oocyte recovery from antral follicles was performed, and oocytes were matured for 24-48 hr in TCM 199 containing 20% patient's own serum, 25mM pyruvic acid and 75mIU/ml hMG. Chian *et al.* (1999a,b) claimed that priming with hCG prior to oocyte recovery increased the developmental competence of the immature oocytes, however Trounson *et al.* (2001) argued that no data were provided to show that the oocytes were immature after hCG injection. Furthermore, in 2000 Chian *et al.* demonstrated that 46% of oocytes were already maturing in hCG-primed patients, and oocytes completed meiosis 12-24 h before oocytes from unprimed patients. There was no significant difference between final fertilization, embryo development and pregnancy rates for hCG primed and unprimed patients (Trounson *et al.*, 2001).

Clinical pregnancy rates of 27.1% (23/85) established from immature oocytes retrieved from unstimulated patients with PCOS following IVM (Cha *et al.*, 2001), along with data from others (Trounson *et al.*, 1994; Chian *et al.*, 2001) suggest that women with PCOS-related infertility could be treated with immature oocyte retrieval, IVM and fertilization.

The application of human oocyte maturation in vitro remains a suitable alternative to surgery or diathermy of ovaries for the establishment of pregnancy in infertile women with PCOS. It is clear that improvements can be made to the maturation conditions for retention of the potential developmental competence of immature oocytes and this

aim should continue to be a priority for research on oocyte maturation (Trounson *et al.*, 2001).

1.3.8 The expectations and limitations of IVM as a method of treatment compared to conventional IVF treatment.

Immature oocyte retrieval combined with IVM could offer an alternative to the current ovarian stimulation protocols used in IVF, however, efficient maturation, fertilisation and embryo development are needed before IVM can be applied routinely in the treatment of human infertility.

1.3.8.1 Potential benefits of IVM

There are many potential benefits of developing an effective IVM programme as an alternative clinical strategy to conventional IVF (Wynn *et al.*, 1998). Approximately 10-15% of oocytes recovered after ovarian stimulation with gonadotrophins (hMG, FSH and hCG) for IVF are immature (Cha and Chian, 1998) requiring further maturation in vitro. IVM might allow such oocytes to be rescued and lead to an improvement in fertilization, embryo cleavage and clinical pregnancy rates. IVM would therefore, reduce wastage of immature oocytes obtained during routine IVF treatment (Van-Steirteghem *et al.*, 1993) and would particularly benefit patients with an unsynchronized cohort of follicles who have a high proportion of immature oocytes collected after stimulation.

IVM would minimize the need for gonadotrophin stimulation of the ovary, which may even become redundant, hence greatly reducing the cost of IVF treatment (Salha *et al.*, 1998). An approximate cost of ovarian stimulation drugs for one cycle of IVF is £800-1500. A further reduction of cost would be contributed by the greatly reduced ultrasound monitoring required in the absence of gonadotrophin stimulation.

IVM might particularly benefit patients diagnosed with PCO and coinciding infertility. Such patients often over-respond to the standard gonadotrophin stimulation normally used for IVF, potentially resulting in OHSS (Rizk and Smitz, 1992; MacDougall *et al.*, 1993). Whilst mild OHSS is of little clinical relevance (Rizk and Aboulghar, 1999), severe OHSS characterised by massive ovarian enlargement with

fluid accumulation in the abdominal and pleural cavities, gross changes in the blood chemistry is a life threatening complication with a risk of thrombosis and death (Brinsden *et al.*, 1998). The risk of OHSS could therefore be minimized not only for high-risk patients (i.e. women with PCO) but also for other patients (Smitz *et al.*, 2001). IVM treatment may therefore be associated with reduced health risks. It may also offer increased patient convenience, through reduced need for blood tests, ultrasonographic monitoring and avoidance of daily injections, compared with conventional IVF treatment in which these are a major factor (Abdul-Jalil *et al.*, 2001). Additionally, side effects reported for ovulation induction treatment such as abdominal bloating, nausea and mood swings would not be encountered with IVM of oocytes from unstimulated cycles.

The potentially increased variety of sources of oocytes for donation afforded by IVM would potentially benefit young women lacking their own oocytes due to premature menopause (e.g. Turners syndrome; familial premature menopause) or definitive sterility (post-oncotherapy or post-surgery for severe endometriosis). Immature oocytes can be recovered from patients undergoing caesarean section (Hwu *et al.*, 1998) and oophorectomy (Cha and Chian, 1998) and matured in vitro, increasing the availability of oocytes for clinical application and research. A combination of immature oocyte cyropreservation, IVM and IVF could provide the opportunity to establish oocyte banks.

Both immature and mature oocytes can be successfully cryopreserved, however, the success of embryo development subsequently is variable and further research is required. IVM might facilitate immediate collection of oocytes in women with cancer or other malignant conditions who cannot spend time undergoing a normal IVF cycle prior to sterilising chemotherapy, or in whom ovarian stimulation is contraindicated. Immature oocyte cryopreservation for subsequent IVM has the potential to overcome many of the legal and ethical problems associated with embryo cryopreservation. 'Custody' and disposal of cryopreserved embryos are sometimes problematic if a couple divorces or a partner dies. The risks of creating embryos and losing a mother are greater if the woman is suffering from cancer and has undergone IVF and stored embryos to preserve some prospects of fertility (Apperley and Reddy, 1995).

However, most patients do not have the time to undergo IVF prior to aggressive cancer treatment. The procedure of IVF and subsequent embryo cyropreservation is inappropriate for children, and unacceptable to many single women who reject donor sperm as an alternative (Oktay and Gosden, 1999).

IVM may also provide a valuable model for investigating the causes of meiotic aberrations and aneuploidies, which are common in mature human oocytes (Gras *et al.*, 1992; Delhanty *et al.*, 1997; Wynn *et al.*, 1998).

1.3.8.2 Limitations of IVM

At present, routine clinical practice relies upon the in vivo environment, augmented by drugs, to achieve the optimum follicular response and maturation of oocytes, since experience has shown the present unreliability of maturing oocytes in vitro whilst retaining developmental competence. The primary problem in oocytes matured in vitro is reduced developmental competence, particularly cleavage and development beyond the 4-cell stage (Trounson *et al.*, 1994). However, an increase in developmental potential may result if the initiation of maturation is triggered in vivo (Chian *et al.*, 1999b).

In a stimulated cycle of IVF, there are an increased number of oocytes and thus an increased number of embryos that are available for embryo transfer compared to unstimulated IVM. Conventional IVF offers a predictable response over the less predictable outcome of oocyte collection and subsequent IVM in natural cycles. Asynchrony of the endometrium with the embryo at the time when transfer might normally be planned is a potential problem with removing and maturing oocytes at various stages of the menstrual cycle. It is anticipated that adequate endometrial preparation through steroidal priming with estrogen and luteal supplementation with progesterone as demonstrated by Russell *et al.* (1997) would help produce a receptive uterine environment in a short period of time, essential to support the establishment and maintenance of a pregnancy. Alternatively, embryos derived from in vitro matured oocytes could be cryopreserved and transferred in a later cycle; however, loss of viability during cryopreservation may further reduce the chance of pregnancy. In donor oocyte programmes, standard hormone replacement schemes are used to

overcome endometrial asynchrony and ensure endometrial receptivity (Cha *et al.*, 1991).

There are potential risks attached to prolonged culture associated with IVM, not encountered with conventional IVF, for example large offspring syndrome (section 1.3.1), observed in ruminants. Further information is required before confident reassurance on this point can be given, although such a syndrome has not been observed to date in human IVM offspring.

Despite the limitations of IVM, the potential benefits of IVM compared to conventional IVF, together with the developing knowledge on mechanisms controlling follicle and oocyte growth and early embryology suggest that the time is right for IVM to be applied with caution in patient treatment.

1.4 Polycystic ovaries

1.4.1 Definition and diagnosis

The term polycystic ovary (PCO), originally known as Stein-Leventhal ovary (Stein and Leventhal, 1935), is used to describe the main phenotypic features of the clinical state known as polycystic ovarian syndrome (PCOS) in the human (Almahbobi *et al.*, 1996). In 1935, Stein and Leventhal described the association of enlarged sclerocystic ovaries with amenorrhoea, infertility and hirsuitism, due in part to the marked heterogeneity of its clinical and endocrine features (Stein and Leventhal, 1935). A universally agreed definition of the polycystic ovary or PCOS is not available (Balen *et al.*, 1999). However, polycystic ovaries are usually enlarged (>9ml) (Franks *et al.*, 1992), containing in one plane at least ten follicles between 2 and 8mm in diameter, arranged peripherally around a dense core of ovarian stroma or scattered throughout an increased amount of stroma (Adams *et al.*, 1985; Adams *et al.*, 1986; Michelmore, 2000).

Surgical procedures such as laparoscopy, laparotomy or wedge resection of the ovaries were originally used to confirm the presence of PCO (Swanson *et al.*, 1981). However diagnostic ultrasonography was introduced in the early 1980s (Swanson *et*

al., 1981; Wild *et al.*, 2000). Transvaginal ultrasonography is now the diagnostic tool of choice for ovarian morphological assessment (Zaidi, 2000).

PCO describes the morphological appearance of the ovary, whereas PCOS describes the clinical condition when polycystic ovaries are found in association with menstrual disturbance, most commonly oligomenorrhoea, the complications of hyperandrogenism (seborrhoea, acne and hirsuitism) and obesity (Balen *et al.*, 1999). Polycystic ovaries can exist without clinical signs of the syndrome, which may be expressed over time.

1.4.2 Incidence

In 1988, Polson *et al.* reported a prevalence of polycystic ovaries of 22% in a 'normal' population. Others (Clayton *et al.*, 1992; Farquhar *et al.*, 1994) have since confirmed the prevalence figure to be approximately 20% in the normal adult female population.

The prevalence in patients referred for IVF is not as clear, although a study carried out by MacDougall *et al.* (1994) in 30 patients referred for natural cycle IVF, all with regular ovulatory menstrual cycles, found 43.5% had PCO.

There are few data in the literature, which address the question of the frequency of PCO in women who present with anovulatory infertility (Franks *et al.*, 1988). In 1986, Adams *et al.* studied an unselected series of 73 women with amenorrhoea and 75 with oligomenorrhoea presenting to a gynaecological endocrine clinic. After ultrasound examination of the ovaries as well as endocrine investigation, PCO were found in 26% of women presenting with amenorrhoea and in 87% of those with oligomenorrhea. The diagnosis of PCO was made primarily on the basis of ultrasound, with at least one other clinical or biochemical marker in > 90% of cases. Adams *et al.* (1986) concluded that PCO are very common in anovulatory women (57%) and are not necessarily associated with hirsuitism or a raised serum LH concentration.

In 1999, Kousta *et al.* studied the prevalence of PCO in women with infertility and compared the endocrine profiles of women with and without PCO within infertility groups and with control subjects with and without PCO. Among 289 couples classified into four diagnostic categories, PCO were found in 83% (81/98) of anovulatory patients, 53% (40/76) of patients whose partner had sperm dysfunction, 50% (26/52) patients with tubal disease and in 44% (28/63) of patients with unexplained infertility. All PCO women included in the tubal disease, sperm dysfunction and unexplained infertility groups were, by definition, ovulatory and had regular cycles. By comparison, PCO was found in 28% (19/67) of a control group of parous volunteers. The prevalence of PCO was significantly higher in the tubal disease group and in the sperm dysfunction group compared to the control group. Although the prevalence of PCO appeared to be higher (44%) in women presenting with unexplained infertility than that of the control group (28%), the difference was not significant. Ovulatory PCO women in all four infertility categories had higher testosterone concentrations in comparison with PCO women of the control group. Kousta *et al.* (1999) suggested that PCO might, perhaps through a hyperandrogenaemic effect, possibly exert an adverse effect on the endometrium, contributing to the causes of subfertility in PCO women with regular menses.

1.4.3 Pathogenesis of PCO

The exact aetiology of PCO remains unknown (Homburg, 1996; Zaidi, 2000). The clinical presentation of women with polycystic ovaries is considerably diverse, ranging from an incidental finding of polycystic ovaries on pelvic ultrasonography in an asymptomatic subject to the 'classic' symptoms of hyperandrogenic anovulation (Franks, 1995; 1997). Those between these extremes may have anovulatory infertility without hirsuitism, or hirsuitism with regular, ovulatory menses (Adams *et al.*, 1986; Franks, 1989, 1997; Dewailly, 2000). Endocrine disturbances (elevated serum concentrations of LH, testosterone, insulin and prolactin are common in women with PCOS (Balen *et al.*, 1999).

All groups of women with a polycystic morphology of the ovaries on ultrasonography are characterized biochemically by elevated serum concentrations of testosterone, regardless of symptoms (Franks, 1991; Franks *et al.*, 2000). Although the highest

testosterone concentrations are found in women with classic PCOS, the mean serum testosterone concentration is also significantly higher than normal in women with PCO who have mild or no symptoms (Franks, 1991). As suggested by Franks (1997), it is unlikely that there is a single cause of the polycystic ovary syndrome due to the heterogeneity of clinical and biochemical features of women with polycystic ovaries. It is more likely that PCOS represents a complex disorder, caused by genetic and environmental factors (Franks, 1997; 2000; Crosignani and Nicolosi, 2001). It appears to have its origins during adolescence and is thought to be associated with increased weight gain during puberty (Balen and Dunger, 1995; Balen *et al.,* 1999). It is not known at what age PCO first appear, although Bridges *et al.* (1993) found that PCO could be detected as early as the age of 6 years.

The growing and antral follicles of PCO are approximately doubled in number when compared with normal ovaries and are found at all stages of development (Goldzieher and Green, 1962; Hughesdon, 1982; Goldzieher, 1981; Green and Goldzieher, 1965 and Almahbobi and Trounson, 1996). Follicular development is arrested prior to selection of a dominant follicle (Erickson and Yen, 1984; Gougeon, 1986; Jakimiuk *et al.,* 1998). The selection of a follicle which responds to an appropriate signal (FSH) to grow, become 'dominant' and ovulate is disturbed in women with PCO, resulting in multiple small cysts distending the ovary, most of which contain viable oocytes but within dysfunctional follicles (Balen, 2000).

In 1982, Hughesden suggested that the appearance of PCO was caused by over-recruitment of follicles into the growing phases and an increase in follicle turnover. If this were the case, the supply of primordial follicles would be expected to be prematurely exhausted in women with PCO. Although, to date, this possibility has not been studied in depth, there is no evidence available to indicate that this is the case. The mechanism of the increase in the number of follicles in PCO remains unknown, but it is suggested that follicle growth is abnormal at all stages and that the increase is due to prolonged follicle growth in both ovulatory and anovulatory women with PCO rather than over-recruitment from the primordial stage (Mason, 2000). There may be a greater number of follicles in anovulatory women with PCO, due to retarded growth combining with arrested development and preventing the normal atresia process.

Support for this suggestion by Mason (2000) comes from data indicating that the majority of structures present in anovulatory women with PCO are functional follicles rather than atretic cysts. However, similar to the normal ovary, ovulatory women with PCO have primarily atretic follicles.

1.4.4 Complications with fertility treatment

Infertility in patients with PCO is caused either by PCOS (i.e. failure to ovulate at a normal rate, and/or hypersecretion of LH), or by any other causes of infertility, e.g. tubal blockage, male factor (Hull, 1987) or a combination of both. For women with PCOS (80% of women with anovulatory infertility), ovulation induction is appropriate, but IVF may be necessary for other causes. Patients with PCOS who have failed to conceive after at least six ovulatory cycles are considered to have coexisting 'unexplained' infertility (Balen *et al.*, 1999; Balen, 2000).

After treatment with clomiphene citrate to induce ovulation, 10-15% of patients with PCOS remain anovulatory despite receiving incremental doses of clomiphene (Gürgan *et al.*, 1994). Exogenous gonadotrophin therapy, in a programme of gentle ovarian stimulation is used for those who fail to respond to clomiphene.

Laparoscopic ovarian surgery (ovarian diathermy or laser drilling) has replaced ovarian wedge resection as the surgical treatment for anovulatory women with PCOS who are resistant to clomiphene (Balen, 2000). Laparoscopic ovarian surgery is performed under general anaesthesia. Laser or diathermy is used depending upon the preference of the surgeon and the availability of the equipment. Laparoscopic ovarian diathermy using 40 W for four seconds in four places on each ovary or laser drilling using a laser via laparoscopy set at 15 W for two seconds in 15 places on each ovary can lead to bilateral ovarian activity and ovulation due to a resultant decrease in serum androgen (testosterone and androstenedione). This effect is possibly due to destruction of the androgen-producing ovarian stroma and drainage of follicles with high androgen and inhibin content (Gürgan *et al.*, 1994). Immunoreactive as well as bioreactive LH concentrations are decreased (Sakata *et al.*, 1990; Balen and Jacobs, 1994). Serum FSH concentrations may be increased. It is hypothesized that the response of the ovary to injury leads to a local cascade of growth factors and those

such as IGF-1, which interact with FSH, result in stimulation of follicular growth and the production of the hormone gonadotrophin surge attenuating/inhibitory factor (GnSAF/GnSIF) which leads to a decrease in serum LH concentrations (Balen and Jacobs, 1991). These procedures have been shown to induce ovulation in > 80% of patients, with a normalization of serum LH concentrations and good rates of pregnancy (Gjonnaess, 1984; Balen, 2000).

Anovulatory PCO is the typical form of PCOS and the most common cause of anovulatory infertility; the selection of a dominant growing follicle is blocked (Almahbobi and Trounson, 1996). It has been suggested that increased serum LH concentrations and increased intra-follicular androgen levels are instrumental in the blockage of normal follicular growth (Baird *et al.*, 1977; Yen, 1980; Franks *et al.*, 1988). A relative lack of FSH may contribute to the persistence of anovulation but is unlikely, in isolation, to be a major cause of it (Franks *et al.*, 2000).

Hypersecretion of LH, found in 40% of women with PCOS, is associated with menstrual disturbances and infertility. It appears to result in reduced conception rates and increased rates of miscarriage in both natural and assisted conception, possibly through an adverse effect of LH on oocyte maturation (Adams *et al.*, 1985; Howles *et al.*, 1986; Homburg *et al.*, 1988; Regan *et al.*, 1990; Balen *et al.*, 1993; Balen, 2000). Homburg *et al.* (1988) suggested that when concentrations of LH are high throughout the follicular phase rather than just at the time of the physiological surge, LH may penetrate the follicle and promote premature maturation of the oocyte, thereby resulting in the ovulation of an oocyte that is 'aged'. Such oocytes may be less likely to fertilize or may produce embryos that survive poorly and therefore abort (Howles *et al.*, 1986; Homburg, 1996). Administration of gonadotrophin-releasing hormone agonist (GnRHa) in order to block endogenous gonadotrophin excretion before and during gonadotrophin therapy was employed by Homburg *et al.* (1993) and resulted in halving the miscarriage rate in women with PCOS treated for induction of ovulation and ovarian stimulation for IVF. In addition, Ashkenazi *et al.* (1995) demonstrated that the oocytes of patients with PCOS who were exposed to GnRHa had a significantly higher implantation rate than those from patients treated only with FSH or HMG in oocyte retrieval cycles (Sengoku *et al.*, 1997).

The polycystic ovary often shows a rapid and intense response to stimulation for ovulation induction, producing a multifollicular rather than a unifollicular response and OHSS is a well-documented complication of induction of ovulation for anovulatory infertility or ovarian stimulation in assisted reproduction in women with PCOS (Franks *et al.,* 1988; Armar, *et al.,* 1990; Dor *et al.,* 1990; Smitz *et al.,* 1991; MacDougall *et al.,* 1992; MacDougall *et al.,* 1993; Almahbobi and Trounson, 1996; Ludwig *et al.,* 1999; Agrawal, 2000).

OHSS is an iatrogenic complication of ovulation induction by gonadotrophins and arises from an excessive ovarian response. The syndrome is characterized by leakage of fluid from the intravascular compartment, with accumulation of protein rich fluid in the peritoneal and eventually pleural cavities, resulting in hypotension and a decrease in renal blood flow and volume of urine. VEGF and other cytokines are pivotal in the pathogenesis of OHSS (Risk and Aboulghar, 1999; Agrawal, 2000).

There are several explanations for the 'explosive' nature of the polycystic ovarian response. Firstly, there are many partially developed follicles in the polycystic ovary, which are readily stimulated to give rise to the typical multifollicular response. Thecal hyperplasia provides large amounts of substrate (androstenedione and testosterone), for oestrogen production. Aromatase, under the influence of FSH, acts on the large amounts of substrate to produce increased amounts of intraovarian oestrogen. The increased numbers of ovarian follicles in PCO are increasingly sensitive to FSH, resulting in multiple follicular development (Balen, 1999).

Zaidi (2000) suggested that increased ovarian stromal blood flow within the polycystic ovary might help to explain the increased ovarian responsiveness that women with PCO show in response to administration of gonadotrophins. Vascular endothelial growth factor (VEGF) is normally confined to the blood vessels in the ovary and is responsible for the invasion of the Graafian follicle by blood vessels after ovulation. VEGF is expressed as a result of the LH surge and is an intermadiate in the formation of the corpus luteum (Ferrara *et al.,* 1998). VEGF is involved in the pathophysiology of OHSS and is widely expressed in theca cells in the increased stroma of polycystic ovaries. The unifollicular response of the normal ovary is

enabled by diversion of blood flow within the ovaries, first from the non-dominant to the dominant ovary and, second, from cohort follicles to the dominant follicle resulting in diversion of FSH away from the cohort follicles, permitting them to undergo atresia (Balen *et al.,* 1999). Agrawal *et al.* (1998) suggested that the widespread distribution of VEGF within the stroma of the polycystic ovary might result in a failure of diversion of blood flow away from the cohort follicles to the leading follicle, leaving many small and intermediate sized follicles, which remain ready to respond to gonadotrophin stimulation.

1.5 Epidermal growth factor (EGF)

1.5.1 Discovery

Cohen *et al.* first described EGF in 1962, and isolated it from mouse submaxillary glands. Human EGF was subsequently isolated from human urine (Starkey *et al.,* 1975; Cohen and Carpenter, 1975) based on its ability to inhibit gastric secretion and was initially called urogastrone (Gregory, 1975; Gregory and Willshire, 1975; Oka *et al.,* 1983).

1.5.2 Structure and Synthesis

Human EGF is a member of the peptide growth factor family (Cooke *et al.,* 1987) and is a single polypeptide chain of 53 amino-acid residues; it is a relatively small molecule (~6 kDa), likely to traverse easily through the zona of human oocytes to exert its effect directly on the oocyte (Goud *et al.,* 1998). EGF was first characterized and sequenced in 1972 (Savage *et al.,* 1972). Since the discovery of EGF, more than 300 EGF-like sequences have been identified, mostly as domains of larger proteins (Barnham *et al.,* 1998). These proteins have been associated with a wide range of functions, including blood coagulation, fibrinolysis, neural development and cell adhesion (Campbell and Bork, 1993; Barnham *et al.,* 1998).

EGF and EGF-like molecules contain three disulphide bridges. Other common structural features are two antiparallel beta-sheets, consisting of a larger triple-stranded beta sheet where residues at the N-terminus contribute a small third strand, and a smaller two-stranded beta-sheet near the C-terminus of the molecule. The three-disulphide bridges anchor the amino-terminal strand and loop, as well as the C-

terminal domain containing the smaller sheet, to the surface of the major beta-sheet (Barnham *et al.*, 1998).

EGF is derived from a precursor of 1,217 amino acids that includes at least seven EGF-like sequences; mRNA for prepro-EGF has been identified in the primordial follicles of the newborn female, demonstrating that the ovary has the capability to synthesize the precursor message for EGF (Rall *et al.*, 1985). EGF is synthesized in small human antral follicles (Westergaard *et al.*, 1990)

The gene coding for EGF has been localized to human chromosome 4, region q25-27 (Morton *et al.*, 1986). It shows mitogenic effects in a variety of mesodermal and ectodermal tissues and is involved in regulating cell proliferation in mammals (Reeka *et al.*, 1998). In the ovary, EGF may regulate the function of follicular development (Das *et al.*, 1991; Lonergan *et al.*, 1996; Goud *et al.*, 1998; Qu *et al.*, 2000). EGF has been previously suggested to play a role in oocyte maturation in vitro (Gomez *et al.*, 1993a; Singh *et al.*, 1997; Goud *et al.*, 1998).

TGF-α consists of 50 amino-acid residues and has 35-40% homology with EGF (Fisher and Lakshmanan, 1990). Like EGF, TGF-α is a potent angiogenic agent (Yeh *et al.*, 1993); it modulates granulosa cell steroidogenesis and participates in follicular maturation (Reeka *et al.*, 1998). Both peptides are secreted as large inactive precursors, which become biologically active following proteolytic cleavage. Despite being products of unlinked genes that are independently regulated, TGF-α and EGF both bind to a single EGF receptor, a 170-kDa transmembrane glycoprotein with tyrosine kinase activity, located on the surface of cells (Gill *et al.*, 1990).

The EGF receptor is found in a wide range of adult tissue types; liver parenchymal cells, fibroblasts and many different epithelial cells all express the EGF receptor (Wiley *et al.*, 1992). In 1992, Wiley *et al.* hypothesized that there may be a heterogeneous population of EGF "receptors" that share a common epitope(s), but differ in structure, binding activities and participation in cell membrane events during pre-implantation development. The EGF receptor belongs to the family of tyrosine kinase growth factor receptors. The EGF-receptor gene is located on human

chromosome 7 in the p13-q22 region (Kondo *et al.,* 1983). It consists of an extracellular ligand binding domain, a single transmembrane region and a cytoplasmic intrinsic tyrosine kinase domain (see Figure 1.5). EGF, TGF-α, vaccinia virus growth factor and other ligands bind to the extracellular domain of the EGF receptor and cause autophosphorylation of five intracellular tyrosine kinases. Autophosphorylation provides anchoring sites for SH2 and phosphotyrosine binding proteins, which in turn lead to stimulation of intracellular Ca^{2+} mobilization and many signal transduction pathway targets including mitogen-activated protein (MAP) kinases, protein kinase-C (PKCs) and phospolipase C-γ. The autophosphorylation also enables other substrates to gain access to the receptor's kinase active site (Panvera, 2001).

The EGF receptor is present in the ovary and the corpus luteum (Khan-Dawood, 1987; Pellicer *et al.*, 1992; Maruo *et al.,* 1993), in the endometrium (Imai *et al.,* 1995), on the oocytes of human primordial (Qu *et al.,* 2000), primary and pre-antral follicles (Maruo *et al.,* 1993; Tamura *et al.,* 1995) and granulosa cells of antral follicles (Reeka *et al.,* 1998).

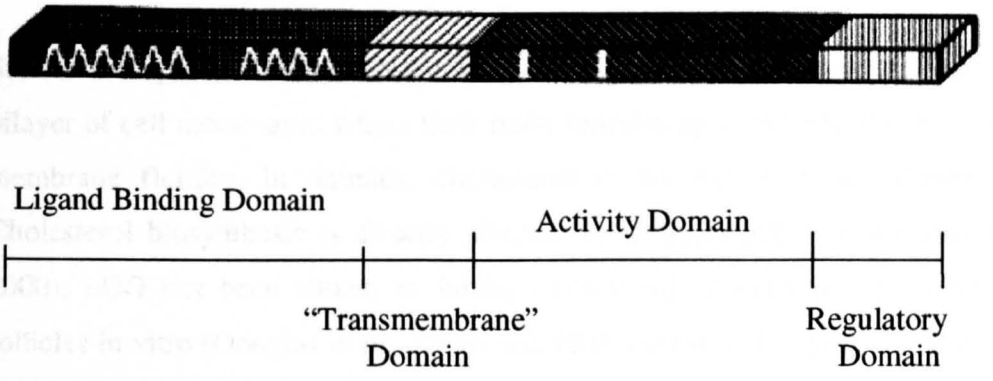


Figure 1.5 Linear view of the EGF receptor protein.
Functional domains are indicated; zigzag lines indicate cysteine-rich regions. (Taken from Gill, 1990).

1.6 Meiosis activating sterol (MAS)

1.6.1 Discovery

In 1985, Westergaard *et al.* suggested that resumption of meiosis in the preovulatory oocyte is triggered by meiosis-inducing substances (MIS) in follicular fluid, and furthermore follicular MIS may be a factor in determining the success of IVF and early embryonic development.

After many years of work, Byskov *et al.* (1995) discovered an active substance, which was termed meiosis-inducing sterol in view of its action to induce resumption of meiosis in vitro as demonstrated in hypoxanthine-arrested cumulus enclosed and denuded mouse oocytes. Spent media from cultured human and bull testes contained the active substance that initiated meiosis in germ cells from fetal mouse testes, which had been cultured for 6 days in the spent medium (Byskov *et al.,* 1998). Human follicular fluid obtained after stimulation with gonadotrophins was found to have a similar effect.

Two naturally occurring sterols, which induce meiotic resumption of both naked and cumulus-enclosed mouse oocytes in vitro have been characterized: FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol) a trienol, extracted from human preovulatory follicular fluid, and T-MAS (4,4-dimethyl-5α-cholest-8,24-diene-3β-ol) a dienol extracted from bull testicular tissue (Byskov *et al.,* 1998).

Sterols are synthesized by most eukaryotic organisms and are present in the lipid bilayer of cell membranes where their main function appears to be the modulation of membrane fluidity. In animals, cholesterol is the major sterol (Mercer, 1993). Cholesterol biosynthesis is directly affected by gonadotrophins (Leonardsen *et al.,* 2000); hCG has been shown to inhibit cholesterol biosynthesis in ovine ovarian follicles in vitro (Douglas *et al.,* 1978) and FSH increases the synthesis and stores of cholesterol in porcine granulosa cells (Baranao and Hammond, 1986). Endogenous gonadotrophin-stimulated steroidogenesis by the pre-ovulatory follicle affects enzyme activity involved in sterol and steroid synthesis; progesterone upregulation is the most well characterized effect (Gore-Langton and Armstrong, 1994; Leonardsen *et al.,* 2000). Within the preovulatory follicle, timely resumption of oocyte meiosis has been

hypothesized to arise from the combined effect of an altered sterol biosynthesis by the granulosa and cumulus cells, resulting in the accumulation of MAS, and a direct transfer of MAS from the cumulus cells to the oocyte via the gap junctional complexes. If this altered enzyme activity is not induced in the cumulus cells, exogenous MAS is probably immediately converted to cholesterol and other steroids (Leonardsen *et al.*, 2000).

Intact connections between cumulus cells and the oocyte of mouse cumulus-enclosed oocytes are essential for FSH (Dekel and Beers, 1978; Downs *et al.*, 1988; Byskov *et al.*, 1997) and forskolin (Guoliang *et al.*, 1994) to initiate the production of meiosis-activating substance by the cumulus cells (Leonardsen *et al.*, 2000), however they are not required for its action upon mouse oocytes (Byskov *et al.*, 1995).

In 1998, Byskov *et al.* hypothesized that resumption of oocyte meiosis is the result of oocyte-dependent FSH-induced FF-MAS production by the cumulus cells which, when transferred to the oocyte, overcomes follicular inhibition of meiosis.

1.6.2 Structure and Synthesis.

FF-MAS and T-MAS are naturally occurring C-29 lipids. MAS are intermediates in the cholesterol biosynthetic pathway and are therefore present in all cells, which produce cholesterol de novo and from lanosterol. However, MAS accumulate only in the gonads (Byskov *et al.*, 1998). T-MAS is the main type of MAS found in adult mammalian testes with trace amounts of FF-MAS. In human pre-ovulatory follicular fluid, FF-MAS is present at a concentration of approximately 1.3μM and T-MAS at about half of this concentration (Byskov *et al.*, 1999). Figure 1.6 shows structural diagrams of FF-MAS and T-MAS.

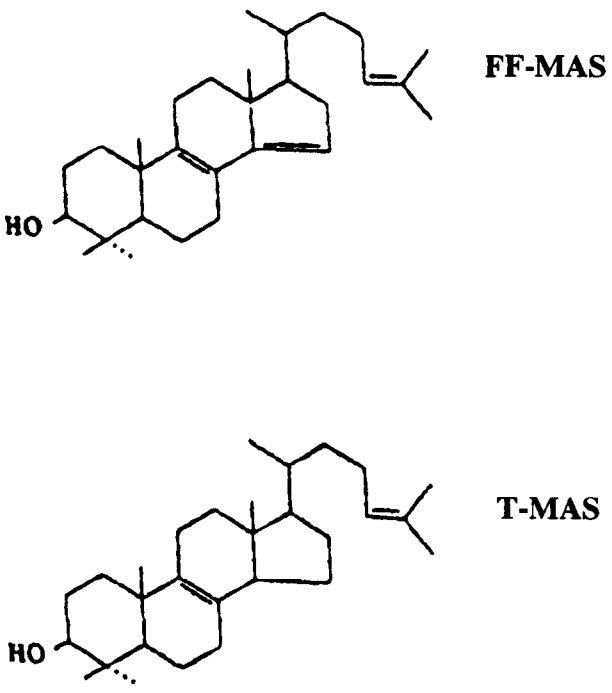


Figure 1.6 Structural diagrams of FF-MAS and T-MAS.

MAS were isolated and purified in three consecutive HPLC steps and all fractions were tested for any meiosis-activating effect on hypoxanthine-arrested mouse cumulus enclosed and naked oocytes. Mass spectrometry and nuclear magnetic resonance were employed to characterize the molecular structure of closely related MAS molecules. FF-MAS and T-MAS are very similar with respect to molecular structure and in their effects in bioassays, suggesting that a family of MAS may exist (Byskov *et al.*, 1998).

FF- MAS and T-MAS are synthesized by cytochrome P450 14α-demethylase (P450-14DM), which converts lanosterol to FF-MAS and sterol Δ14-reductase (Δ14R), reduces FF-MAS to T-MAS (Schroepfer *et al.*, 1982) (Figure 1.7).

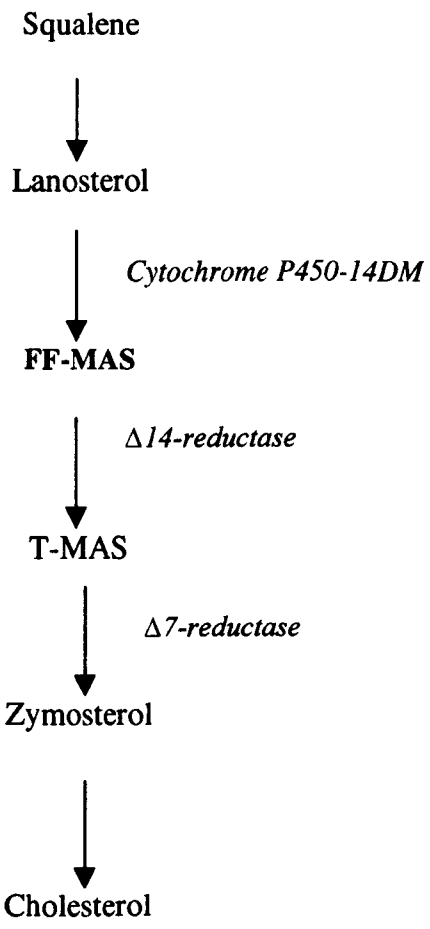


Figure 1.7. MAS intermediates in the biosynthesis of cholesterol from lanosterol. The enzymes are indicated in *italics* (Modified from Byskov *et al.*, 1998).

Several studies have been carried out using purified or synthetic FF-MAS to evaluate its effectiveness in stimulating GVBD in meiotically arrested oocytes. Leonardsen *et al.* (2000) showed that blockade of the enzyme Δ14-reductase, using an inhibitor (AY9944; a drug used to lower plasma cholesterol by inhibiting enzymes involved in cholesterol biosynthesis) in hypoxanthine–arrested mouse cumulus-oocyte complexes resulted in the accumulation of endogenously produced FF-MAS in the cumulus cells and the resumption of oocyte meiosis. The same effect was not observed in naked oocytes, leading Leonardsen *et al.* (2000) to propose that MAS biosynthesis occurs in the cumulus cells of cumulus-oocyte complexes (COC) and not in the oocyte itself, and that this may be responsible for resumption of meiosis in oocytes in vivo.

Blocking downstream metabolism of FF-MAS with AY9944-A7 stimulated GVBD of COC in a dose-dependent manner, but not denuded oocytes. Concentrations of 5, 10 and 25μmol AY9944-A7 1^{-1} increased GVBD in COC significantly compared with the controls. At 5 and 10μg/ml, FF-MAS induced a significant dose-related increase in the percentage of GVBD in naked oocytes, however 5μg/ml, FF-MAS had no effect on COC, whereas 10μg/ml, FF-MAS significantly stimulated percentage of GVBD.

Alternatively, Downs *et al.* (2001) proposed that induction of meiotic maturation by inhibitors of enzymes situated downstream of FF-MAS in the sterol biosynthetic pathway, such as AY9944 (as used by Leonardsen *et al.*, 2000) may be acting by preventing accumulation of inhibitory downstream metabolites, for example cholesterol. This is a plausible consideration, since AY9944 has an additional site of action on Δ7-reductase that produces cholesterol from 7-dehydrocholesterol. Indeed, cholesterol was shown to augment the inhibitory effect of dbcAMP in both cumulus-enclosed and denuded oocytes in a dose-dependant fashion.

Ketoconazole another widely used enzyme inhibitor, inhibits the Cytochrome P450-14DM enzyme, which converts lanosterol to FF-MAS in vitro (Yoshida *et al.*, 1996). In 1998, Tsafriri *et al.* reported no effect of ketoconazole (a potent inhibitor of sterol synthesis) on oocyte maturation in the rat, both in in vivo gonadotrophin-stimulated oocytes and spontaneously maturing oocytes in vitro. Stimulation of P450-14DM by gonadotrophins opposes an inhibitory effect by ketoconazole on this enzyme in the living animal (Byskov *et al.*, 1999). Spontaneous meiotic maturation and FSH-induced maturation are mediated by different intracellular pathways (Coticchio and Flemming, 1998). Similarly, it has been suggested that FSH-induced maturation is mediated by the generation of a stimulatory signal, which overcomes an inhibitory input from the follicle, however; spontaneous maturation does not require a stimulatory signal (Downs *et al.*, 1993). There is a similar difference between MAS-induced and spontaneous maturation, which may explain how rat oocytes mature spontaneously despite an inhibitory action of ketoconazole on MAS biosynthesis. (Leonardsen *et al*, 2000). When ketoconazole was tested on FSH-induced maturation by Downs *et al.* (2001) it was found to have inconsistent effects on meiotic

resumption. Mouse cumulus enclosed oocytes (CEO) were cultured 17-18 hr in medium containing either 300μM dbc-AMP ± FSH or 4 mM hypoxanthine ± FSH. In dbcAMP-arrested mouse CEO, ketoconazole totally eliminated FSH-induced maturation (significant inhibition at 5μM); but only minor suppression of meiotic induction occurred in hypoxanthine-arrested CEO, and considerably higher concentrations of ketoconazole (30μM) were required for this effect. This led Downs *et al.* (2001) to suggest that the effect of ketoconazole on meiotic maturation is unrelated to FF-MAS levels.

The signalling pathways for FF-MAS during resumption of oocyte meiosis have not yet been clarified. The MAS receptor has not yet been identified, although Janowski *et al.* (1996) observed FF-MAS to be a ligand for the orphan nuclear receptor LXR-α, and demonstrated transactivation of the receptor by several oxysterols, including FF-MAS. However, results by Grondahl *et al.* (1998) found that none of these oxysterols or cholesterol were active in inducing resumption of meiosis in hypoxanthine–arrested mouse oocytes, and consequently no correlation was observed between meiosis stimulation and LXR-α activation. This led Grondahl *et al.* (1998) to conclude that it is not likely that the LXR-α represents the MAS receptor responsible for resumption of meiosis in oocytes. However, a putative nuclear MAS receptor could have similarities at least in the ligand-binding domain to LXR-α (Grondahl *et al.*, 2000).

According to Faerge *et al.* (2001) specific binding of FF-MAS is predominant at the oolemma of denuded oocytes, suggesting the existence of a plasma membrane-associated molecule with affinity for FF-MAS (i.e., a putative FF-MAS receptor).

In 2000, Grondahl *et al.* set out to elucidate whether the MAS-induced oocyte maturation-signalling pathway involved transcription or translation. This was determined by culturing arrested immature mouse oocytes with either the protein synthesis inhibitor cycloheximide or the heteronuclear RNA inhibitors: α-amanitin or actinomycin D. They demonstrated that protein synthesis was essential for maintaining the oocyte's responsiveness to MAS-induced resumption of meiosis however; transcription was not required. It was suggested that either a certain level of

protein synthesis is essential for the MAS signalling pathway, or that a rapid receptor turnover makes it essential with an ongoing de novo protein synthesis inside the oocyte (Grondahl *et al.*, 2000).

1.7 Project aims and objectives

IVM of human oocytes has considerable scope for improvement, both in relation to the technique itself and the pregnancy rate after transfer of embryos resulting from IVM of oocytes. Such improvements could result in major benefits for a range of patients facing infertility treatment. However, these must be based upon an improved understanding of the events controlling oocyte development, particularly in the final pre-fertilization stage, known as maturation.

Various factors are known to affect oocyte maturation, having effects expressed at different times after exposure, although an optimal culture system for human IVM is yet to be developed. A successful IVM protocol that is inexpensive both in labour and materials whilst overcoming one of the factors currently limiting oocyte maturation in vitro, namely poor subsequent developmental competence, will provide a major breakthrough in ART.

This project aimed to provide a sound basis of scientific information gained through methods compatible with the clinical application of IVM, in order to provide evidence to inform and facilitate the eventual clinical application of IVM. This project sought to address the specific issue of developmental competence of embryos arising from in vitro matured oocytes, which has, to date, limited the use of immature oocytes in human infertility treatment. Throughout this project, factors having the potential to affect developmental competence in various ways have been assessed using human oocytes, since the extrapolation of data from animals to humans in this field has proven unreliable. The government's regulatory authority licensing research and treatment of infertility (HFEA) considered the available information worldwide in humans and animals to be insufficient information upon which to base a clinical trial, requiring further evidence of the safety and efficacy of the process before permitting its application in the UK. This project was designed in order to make a direct

contribution to this process and has been broadly welcomed for its focus on human material.

The project aimed to assess specific factors affecting in vitro maturation of human oocytes in relation to the developmental competence of the resulting embryos. These were:

- The origins of the oocytes in terms of donor characteristics and exposure to ovarian stimulation.
- The effects of supplementation of in vitro maturation culture media with EGF or FF-MAS, both demonstrated in animal studies to have a positive role in oocyte maturation.
- The size, growth and other visible features of oocytes at collection and during maturation in vitro,
- The normality of chromosome and spindle development during in vitro maturation.

In order to assess developmental competence, some in vitro matured oocytes were inseminated to create human research embryos using the technique of injecting a single sperm from a known fertile donor, with the intention of minimizing male factor biases in embryo development, thus enabling the female, oocyte-derived contribution to be determined. The normality of fertilization and cleavage of the resulting embryos was assessed using standard clinical embryological criteria based on morphology as well as image analysis enabling a quantitative evaluation of the oocyte and embryo database. This has been the first UK licensed project permitted to create and culture human embryos specifically for research purposes.

The outcome of this research project has been an improved understanding of the patient groups who may be suitable for eventual IVM treatment, together with information on the embryological parameters of IVM. A number of original observations have been made, including the following important findings: The potential of FF-MAS as a natural stimulator of oocyte maturation has been confirmed in humans, as anticipated from previous studies in mice, and the ongoing growth of human oocytes associated with further development during maturation culture has been presented for the first time. One research paper has been published and a further submission is in preparation. This work has brought the goal of clinical application of

in vitro maturation closer and has contributed novel data to the literature on human oocytes.

Chapter 2

Materials and Methods

2.1 Media preparation

All media preparation was carried out in laminar air flow cabinets using aseptic technique. The media were filtered through sterile 0.22μm filters (Millipore Corp., Bedford, UK) and equilibrated with 5% CO_2 in air at 37°C before use. The compositions of the media used are presented in Appendix II.

2.1.1 5% w/v Human serum albumin (hSA)

20% w/v hSA (Immuno Ltd, Thetford, Norfolk, UK) was diluted 1:3 with sterile water for injection (Fresenius, Kabi Ltd, Warrington, UK) to give a working concentration of 5% w/v, filtered and stored at 4°C.

2.1.2 Earle's balanced salt solution (EBSS)

EBSS (Gibco, Life Technologies, Paisley, UK) was used for equilibrating the mineral oil (Sigma, Poole, Dorset, UK) and for diluting the hyaluronidase (Scandinavian IVF Science, Gothenburg, Sweden) when stripping cumulus cells prior to ICSI.

EBSS was prepared in bulk weekly from 10× concentrate (Gibco), supplemented with sodium pyruvate (0.22mmol/l, Sigma), penicillin (100U/ml) and streptomycin (100μg/ml) at 285mOsm and containing 10% v/v of a 5% w/v injectable preparation of hSA (Immuno Ltd).

This medium was pre-tested using a mouse '2 cell to blastocyst' biotoxicity assay (performed by H. Baker as indicated in acknowledgements).

2.1.3 Phosphate buffered saline (PBS, Gibco)

PBS was used to dilute Hoechst 33258 (Sigma) for fluorescent staining of chromatin (2.5.3) and to dilute the monoclonal anti-α-tubulin antibody for the fluorescent labelling of tubulin (2.7.1).

2.1.4 Follicle flushing medium

2.1.4.1 Patients undergoing transvaginal oocyte retrieval for ICSI treatment

Oocytes were collected transvaginally under ultrasound guidance into prewarmed Ham's F10 medium (ICN Biomedicals, Thame, UK) supplemented with heparin (3IU/ml, Unihep, Leo Laboratories Ltd, Buckinghamshire, UK), which was also used for flushing the follicles.

2.1.4.2 Patients undergoing laparoscopic oocyte retrieval

Follicle flushing medium was prepared on the morning of oocyte retrieval for patients having PCO who were undergoing a voluntary additional procedure of oocyte retrieval during the course of laser drilling or diathermy operations.

Hams F10 medium (ICN Pharmaceuticals Ltd) was supplemented to produce final concentrations of 20mmol/l HEPES, 3IU/ml heparin (Leo Laboratories Ltd) and 0.5% hSA (Immuno Ltd). Sterile 10ml plastic tubes (Falcon; Fahrenheit Laboratory Supplies, Milton Keynes, UK) were prepared for aspirate collection, containing 2ml of follicle flushing medium and the tubes were maintained at 37°C in a thermostatic hot-block (Grant, Cambridge, UK).

The remaining medium in a 250ml flask (Falcon, Fahrenheit Laboratory supplies) was taken to theatre in a portable incubator (Cell Trans 4016 Transport Incubator; Labotect, Hunter Scientific Ltd, Essex, UK) at 37°C to be used for flushing the oocyte collection needle.

2.1.5 In vitro maturation (IVM) medium

IVM medium was prepared on the morning of oocyte retrieval. The medium comprised Medium 199 diluted from M199 2× (Gibco) supplemented with sodium pyruvate (0.23mM, Sigma), penicillin G (50IU/ml) and streptomycin (50µg/ml, Gibco) and 0.5% filtered hSA (Immuno Ltd). The final osmolarity was 326 ± 0.8mOsm.

Tissue culture dishes (40 x 12mm: Nunclon, Life Technologies, Paisley, Scotland) containing 2ml IVM medium were pre-equilibrated in a humidified incubator at 37°C containing 5% CO_2 in air, ready to receive isolated immature oocytes for randomised allocation to further culture.

2.1.6 Sperm storage medium

Cryopreserved donor semen was used for ICSI procedures. After performing a semen analysis according to standard procedures, the liquefied semen was gradually mixed with an equal volume of test yolk buffer cryoprotectant medium (Rochford Medical Ltd, Buckinghamshire, UK) within one hour of production. Mixing was carried out slowly to minimise the osmotic shock to the spermatozoa. 1ml volumes of the semen cryoprotectant mixture were loaded into 1.5ml labelled cryovials (Camlab, Cambridge, UK) and placed into the vapour-freezing vessel, and the vessel was suspended in liquid nitrogen vapour in the neck of a liquid nitrogen storage tank. After 15 minutes, the vials were quickly transferred into a cooled labelled cryocane (Camlab); a cane sleeve was used to cover the cane, which was immersed in liquid nitrogen.

2.1.7 Hyaluronidase

For cumulus-corona removal, hyaluronidase 10× (in commercially prepared bicarbonate and HEPES buffered medium containing hSA: Scandinavian IVF Science, Gothenburg, Sweden) was diluted 1:2 in prepared EBSS (2.1.2), gassed with CO_2 incubated at 37°C for 30 min. Immediately prior to use, 50µl of the hyaluronidase dilution was added to a 100µl drop of EBSS + 0.5% hSA in a prepared tissue culture dish (Falcon, Fahrenheit) with six 100µl drops of medium under ~7ml paraffin oil (Ovoil; Scandinavian IVF Science) previously pre-equilibrated overnight at 37°C containing 5% CO_2 in air before use to give an approximate final concentration of 80 IU/ml.

2.1.8 Minimal essential medium (MEM)

HEPES-buffered MEM (Sigma) supplemented with 10% v/v of a 5% w/v solution of hSA was used for ICSI (2.4.3). MEM contained earles salts, 25mM HEPES and sodium bicarbonate, without L-glutamine.

2.1.9 Embryo Culture medium

2.1.9.1 P1 medium with gentamycin (preimplantation stage one; Irvine Scientific, Santa Ana, CA).

This commercially purchased medium was used for the culture of embryos in the EGF study. It is a synthetic, defined medium for in vitro procedures. It was purchased in 100ml bottles.

P1 medium is bicarbonate based and is modified synthetic human tubal fluid (HTF), it lacks glucose and inorganic phosphates, contains sodium citrate (0.15mg/l), taurine (0.05mM) and gentamycin (10µg/ml), pH 7.1, 292 mOsm.

It was supplemented with 10% synthetic serum substitute (SSS, Irvine Scientific) and used for culture up to the 8-cell stage according to the manufacturers instructions.

Tissue culture dishes (60 x 15mm: Falcon, Fahrenheit) were set up with six 100µl drops of medium under ~7ml of paraffin oil (Ovoil; Scandinavian IVF Science) and pre-equilibrated at 37°C containing 5% CO_2 in air before use.

2.1.9.2 S1 and S2 medium (Scandinavian IVF Science)

These commercially purchased sequential media were used for the culture of embryos in the MAS study. Such sequential media were developed to take into account the changes in embryo physiology and requirements occurring as the embryo develops from the zygote to the blastocyst. S1 is a physiological salt buffer containing hSA, low glucose and essential amino acids intended to support embryo cleavage to 8 cells i.e. pre-compaction.

S2 is Gardner's "blastocyst culture medium" (Gardner *et al.*, 1998), it contains a higher concentration of glucose along with various non-essential amino acids believed to be appropriate for embryo development from 8-cell compaction onwards. S1 and S2 media were purchased in 20ml volumes. Table A6 shows the composition of S1 and S2 media, the amounts of each component were not given, as they remain a commercial secret although the concentrations are presented in descending order down the table.

Tissue culture dishes (Falcon, Fahrenheit) were set up with six 100µl drops of medium under ~7ml of paraffin oil (Ovoil; Scandinavian IVF Science) according to the manufacturer's instructions. S2 dishes were made up early on the day of use using pre-gassed oil.

2.1.9.3 G1 and G2 medium (Scandinavian IVF Science)

In February 1999, a media change was made and G1.2 and G2.2 replaced S1 and S2 respectively. S1 and S2 were withdrawn by the company in favour of G1.2 and G2.2, and were unavailable for five months, until S2 was subsequently made available again as "CCM", due to numerous requests to the company by various groups.

Tissue culture dishes (Falcon) were set up with six 100µl drops of medium under ~7ml paraffin oil (Ovoil; Scandinavian IVF Science) according to manufacturers instructions.

2.1.10 Pre-equilibration of mineral oil used in ICSI procedure

20ml HEPES buffered MEM (Gibco) (minus serum) was added to a 250ml flask (Falcon, Fahrenheit) containing 100ml of mineral oil (Sigma). CO_2 was used to gas the media via a flamed glass Pasteur pipette. The flask was stored with a loosened lid at 37°C with 5% CO_2 in air, for at least four hrs prior to use. Thus ensuring that the mineral oil was at the correct pH and temperature.

2.2 Oocyte maturation in vitro

2.2.1 EGF

2.2.1.1 EGF preparation and culture system

Human recombinant EGF (Calbiochem-Novabiochem Ltd, Nottingham, UK) was used. Sterile 10mM acetic acid was used as the vehicle as recommended by the manufacturer. Final concentrations of 0.1, 1, 10ng/ml EGF and control (no EGF) were set up by addition of 1µl of each stock to 100µl IVM medium drops in tissue culture dishes with non-adherent surface (Falcon, Becton-Dickinson, NY) with ~7ml paraffin oil overlay (Ovoil, Scandinavian IVF Science). The final concentration of acetic acid in all drops of medium including controls was 10µM, which produced no alteration in

pH. Dishes were pre-equilibrated at 37°C in an atmosphere of 5% CO_2 in air on the morning of oocyte retrieval, which took place in the afternoon. Up to three oocytes were co-cultured in each drop

2.2.2. FF-MAS

2.2.1.1 FF-MAS preparation and culture system

FF-MAS was purified from human follicular fluid by high-performance liquid chromatography (HPLC) and detected using a photo-diode array system (Waters, Copenhagen) as described by Byskov *et al.* (1995) and Baltsen and Byskov (1999). (Performed by Mogens Baltsen, as indicated in the acknowledgements).

FF-MAS was stored at -20°C in n-heptane under N_2 in glass vials. Before use, a stream of nitrogen gas was used to evaporate the heptane from a known quantity of MAS, which was dissolved in a known volume of absolute ethanol (EtOH). Calculated volumes were added to prepared IVM media in sterile, washed glass tubes to final concentrations of 10 and 30μg/ml (24.4 and 73.2μM). EtOH alone, treated in the same manner, was added to controls. All wells contained a final concentration of 0.5% EtOH. FF-MAS/EtOH was added to the cultures ~ 1hr before oocyte retrieval. FF-MAS remaining in EtOH was evaporated to dryness and redissolved in n-heptane for storage at -20°C under N_2.

Volumes of 100μl IVM medium containing the various concentrations of FF-MAS were set up in a sterile 96 well plate (Nunclon), which also contained medium in the surrounding wells to increase humidity and reduce evaporation. Cultures were housed in a humidified incubator (37°C, 5% CO_2 in air). An oil overlay was not used, in order to avoid oil phase extraction of FF-MAS. Control experiments demonstrated that the osmolarity of cultures maintained under these conditions varied by <1% after 24 hr. Up to 4 oocytes were co-incubated in each well.

2.3 Source and collection of oocytes

2.3.1 PCO patients

2.3.1.1 Recruitment of patients

These patients were attending gynaecology or infertility clinics at the Walsgrave Hospital. Patients having symptoms of PCO (Adams *et al.*, 1986) and who were undergoing treatments which required a laparoscopy, were invited to participate in the study. The procedure for oocyte retrieval and the nature of the study was explained during the consultation, and patients wishing to participate provided written consent, in accordance with protocols approved by Coventry Research Ethics Committee. Only two patients approached regarding the study, declined to participate.

Inclusion criteria were: (i) PCO diagnosed primarily by the ultrasound appearance of the ovaries, but additionally by a history of oligomenorrhoea or amenorrhoea, and blood hormone measurements potentially including androgen, LH elevation; (ii) no drug therapy for infertility in the four months before surgery; and (iii) requiring diagnostic laparoscopy and/or laser drilling of the ovaries. Laparoscopy was scheduled irrespective of the stage of the menstrual cycle.

2.3.1.2 Theatre Procedure

Oocyte retrieval procedures were carried out in the afternoon in theatres on the same site but separate from the main embryology laboratory. A previously described technique (Trounson *et al.*, 1994) was followed using a purpose-designed 17G needle (Cook IVF, Letchworth, UK) and a laparoscopic approach. All visible follicles were individually punctured and aspirated using 80-100mm Hg suction. Measurement of follicular diameter was not possible by direct laparoscopic inspection since the follicles were relatively small (<10 mm) and the stroma was dense as expected in women with PCO (Adams *et al.*, 1985). The fluid was collected into the 10ml sterile plastic tubes containing follicle-flushing medium prepared in the morning (2.1.4.2). The aspirates were transported to the embryology laboratory in a portable incubator (Cell Trans 4016 Transport Incubator, Labotect). The oocyte aspiration procedure took ~40 minutes and the journey <10 min.

2.3.1.3 Oocyte identification

Follicular aspirates were placed into an Em-Con filter of 75μm pore size (Immuno Systems, Spring Valley, WI, USA) which had been pre-rinsed with warmed follicle flushing medium. The aspirates were flushed with 250ml of follicle flushing medium to remove contaminating blood cells and the filter retentate was transferred to sterile dishes (Falcon, Fahrenheit).

Oocyte-cumulus masses and free oocytes were identified using a dissecting microscope (Leica Microsystems Ltd, Milton Keynes, UK), equipped with a heated stage at 37°C, and transferred to the prepared tissue culture dishes (Nunclon) containing 2ml pre-equilibrated IVM medium.

2.3.1.4 Cumulus grading

The level of cumulus cover surrounding the oocyte was graded subjectively from 0-3, where 0 = devoid of cumulus; 1 = partially covered zona; 2 = completely covered zona; 3 = substantial multi-layered cumulus cover (Figure A2, Appendix III).

2.3.1.5 Oocyte viability and maturity assessment

An oocyte was considered to be viable if it had an intact oolemma, a light coloured cytoplasm and a regular-appearing spherical shape. In previous experiments using the fluorescent vital stains, carboxy-fluorescein diacetate (CFDA) and propidium iodide (PI), these features had been confirmed as identifying viable oocytes. Where possible, it was recorded whether a germinal vesicle (GV) was present in the ooplasm, and any other features of oocyte morphology. Oocytes obscured by tightly surrounding cumulus cells (cumulus enclosed; CE) were presumed to contain a GV. The initial assessment of maturity was performed ~2 hrs after oocyte retrieval. Oocytes, which were clearly atretic on collection, e.g. dark cytoplasm and irregular shape, were noted before discarding.

Images of oocytes were collected daily via a video link on an inverted high power microscope (Olympus IX 70) employing Hoffman optics and Image Pro-plus software (Media Cybernetics).

2.3.1.6 Oocyte allocation

Individual viable oocytes were randomly allocated to either one of the four concentrations of EGF in the EGF study or one of three concentrations of FF-MAS in the FF-MAS study using tables of random numbers. The dishes of oocytes were incubated at 37°C under 5% CO_2 in air and examined again after 23-24 hr.

2.3.2 ICSI patients

2.3.2.1 Recruitment

Women undergoing ICSI at the Centre for Reproductive Medicine, Walsgrave Hospital were approached in advance of oocyte retrieval with information about the study. Women wishing to participate gave written consent prior to the oocyte retrieval using information sheets and consent forms approved by Coventry Research Ethics Committee. Approximately 80% of patients approached, agreed to participate. In the event of consent, any immature oocytes among the cohort collected for ICSI treatment were used in this study.

2.3.2.2 Theatre Procedure

Patient preparation, oocyte retrieval and embryological procedures were performed using the centre's standard protocols for ICSI (Garello *et al.*, 1999).

2.3.2.3 Oocyte identification and randomisation

Any immature oocytes identified when oocytes were stripped of cumulus in preparation for ICSI were randomly allocated to FF-MAS treatment groups using random number tables and the plates were incubated at 37°C under 5% CO_2 in air in a humidified incubator. Thereafter, they were treated identically to oocytes from patients with PCO. Oocytes were considered immature if they contained a germinal vesicle (GV) or had undergone germinal vesicle breakdown (GVBD) but had not released a polar body (pb).

2.3.2.4 Oocyte maturity assessment for ICSI

Oocytes displaying a GV were considered to be arrested at prophase I of meiosis. Those lacking a GV and a pb were considered to have undergone GVBD. Those having a pb were considered mature MII and subjected to ICSI. Invasive analysis to

confirm the stage of meiosis was performed by chromatin staining of all non-matured and unfertilised oocytes as described in section 2.5.3.

2.3.3 Collection of mouse oocytes

Mouse oocytes were used for fluorescent labelling of tubulin and chromatin. Female, F1, B6/C3 mice at ~8 weeks of age were superovulated by intraperitoneal injection with 6-12 IU of pregnant mares serum gonadotrophin (PMSG; Intervet Laboratories Ltd, Cambridge), followed by an injection of 6-12 IU hCG (Intervet Laboratories Ltd) intraperitoneally 48 hrs later (Carried out by Animal House Personnel).

48 hrs after the administration of the hCG injection, the mice were killed by cervical dislocation. Prior to killing the mice, two 35 mm dishes (Nunclon) and a conical tube each containing 1ml HEPES buffered Earle's were set up and kept at 37°C on a heated microscope pad. The conical tube was kept in a humidified incubator (37°C, 5% CO_2) with the lid loose until use. All dissection equipment was sterilised with 70% alcohol and flaming and laid on sterile tissues until use. After killing the mice, the abdomen of the mouse was swabbed with 70% alcohol, before tearing the skin open between the fore and hind limbs. A cut was then made through the peritoneum to expose the abdominal cavity, and locate the two uterine horns. The oviducts, some ovarian tissue and about 5mm of the uterus from each side was dissected out and placed into the culture dish with HEPES buffered Earle's.

Under the dissecting microscope, the bursa was gently torn and the ovarian tissue removed using sterile forceps and scissors. The uterus tissue was cleanly cut where it joined the oviduct, the coils of the oviduct were teased apart and the clean oviduct was moved to a new culture dish. This procedure was repeated for the other oviduct so the two clean oviducts from the same mouse were in a dish together. The oviducts were torn at the swollen points to release the oocyte- cumulus complexes, which were then transferred into the conical tube containing HEPES buffered Earle's. The tube was then transported in a transport incubator (37°C) to the Centre for Reproductive Medicine where it was kept in a humidified incubator (37°C, 5% CO_2) with the lid loose until use.

Oocytes were stripped of cumulus using the protocol described in 2.4.2, although the time left in hyaluronidase was ~60 seconds.

2.4 ICSI

2.4.1 Sperm preparation

Two different methods were employed to recover motile spermatozoa from the frozen ejaculates of two known fertile sperm donors, due to the different characteristics of their samples. The semen samples were frozen in test yolk buffer cryoprotectant medium (Rochford Medical Ltd), section 2.1.6.

Donor 1 sample contained quite a high proportion of immotile sperm and a large degree of cellular debris.

Donor 2 sample contained spermatozoa with good motility; therefore the swim-up technique was employed.

Donor 1- Density gradients composed of Sil-Select (silane–coated colloidal silica in HEPES-buffered EBSS; Fertipro) upper layer (40%) and lower layer (80%) were used, followed by a swim-up procedure.

1ml of 40% upper layer was placed in a sterile 14ml conical tube (Falcon), using a sterile syringe and needle; 1ml of 80% was carefully layered below the 40% layer.

0.5ml EBSS supplemented with 0.5% hSA was added to an aliquot of thawed ejaculate. Approximately 1ml of the semen mixed with EBSS + 0.5% hSA was then layered gently on top of the density gradient using a sterile Pasteur pipette and the tube was centrifuged at 460g for 10 min.

The supernatant was then removed, and the pellet gently resuspended in 1ml EBSS + 0.5% hSA.

<u>Swim-up:</u>

The sperm suspension was then transferred to a new sterile round-bottomed tube (Falcon) and centrifuged at 460g for 5 min. The supernatant was immediately removed from the top of the sample to just above the pellet which was then gently overlaid with ~200µl EBSS + 0.5% hSA. The sample was left at RT for 30 min to allow motile spermatozoa in the sample to "swim up". The supernatant (containing motile spermatozoa) was then removed from the tube without disturbing the pellet; and transferred to a new 5ml sterile tube (Falcon).

After examination of a 10µl aliquot on a Mackler chamber, an appropriate aliquot (~1µl) was transferred to a 5µl droplet of HEPES buffered MEM (minimal essential medium; Gibco) on the pre-prepared ICSI dish (which contained droplets for oocytes and PVP, see section 2.4.3) prior to the ICSI procedure.

Donor 2– ~0.5ml of frozen ejaculate was thawed at RT in a 5ml sterile tube (Falcon); 1ml EBSS + 0.5% hSA was added to the thawed ejaculate before centrifuging at 460g x 5 min. The supernatant was removed and discarded, 100µl of EBSS + 0.5% hSA was added to resuspend the pellet, and a further 100µl of EBSS + 0.5% hSA was carefully layered on top. The sample was incubated at 37°C, 5% CO_2 in air for 30 min to allow motile spermatozoa to swim up.
Prior to the ICSI procedure, a 1µl aliquot was gently removed from the top layer and transferred to a 5µl droplet of HEPES buffered MEM on the pre-prepared ICSI dish.

2.4.2 Removal of Cumulus cells

Where present, the cumulus and corona radiata of oocytes were removed by incubation in EBSS supplemented with 0.5% hSA containing hyaluronidase (80 IU/ml; Scandinavian IVF Science) for ~30 seconds. The oocytes were transferred to droplets of EBSS with 0.5% hSA under paraffin oil overlay (Ovoil). The corona cells were removed by mechanical aspiration using a fine hand drawn sterile Pasteur pipette with a diameter of ~150µm. Oocytes were washed in EBSS with 0.5% hSA through several 100µl droplets under oil and the maturational status observed under an inverted microscope at 200x magnification.

2.4.3 ICSI reagents and set up

Microinjection dishes (50 x 9mm: Falcon 1006) were prepared in advance. Six 5µl drops of HEPES buffered MEM supplemented with 0.5% hSA were placed towards the centre of the dish. One of these for sperm incubation and the remainder for up to five oocytes, one per drop. A 5µl drop of 10% polyvinylpyrrollidone (PVP) (ICSI-100, Scandinavian IVF Science) was placed in the centre of the six drops. The drops were overlaid with ~5ml pre-warmed mineral oil (Sigma), pre-washed with HEPES buffered MEM (Figure 2.1).

Mature oocytes, identified by the presence of a polar body were injected with spermatozoa according to the Centre's established ICSI protocol (Garello *et al.*, 1999)

Prior to performing ICSI on the oocytes in this study, ICSI practice was undertaken to achieve standards required for ICSI license application to the HFEA. After learning how to set up the micromanipulators used for ICSI, consent was obtained from patients to use any oocytes that failed to fertilize during their treatment (surplus to patients' requirements) to practice the injection of latex beads into oocytes. 50 one day-old unfertilised oocytes were injected over a period of time with latex beads and survival was recorded the following day after injection. A survival rate of 82% was achieved.

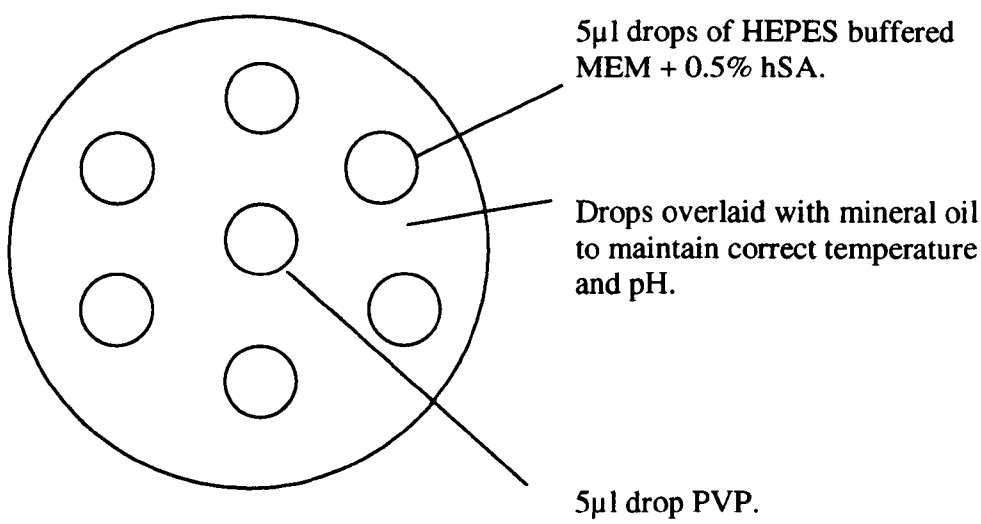


Figure 2.1 Diagram of microinjection dish as seen from above, used for ICSI procedure.
Low sided microinjection dish used was 50 x 9mm (Falcon 1006).

2.5 Embryo culture

2.5.1 Embryo culture media

After injection, oocytes were transferred individually to 100µl drops of either P1 medium with gentamycin (Irvine Scientific), S1 or G1.2 medium (Scandinavian IVF Science) as described in section 2.1.9. Dishes containing embryo culture media were pre-equilibrated at 37°C in an atmosphere of 5% CO_2 in air for 1 day before use. The oocytes were checked for fertilization 14-18 hrs post-injection using standard procedures of pronuclear assessment. Oocytes having two pronuclei and two polar bodies were considered to be normally fertilized. If embryos were still cleaving on day 3, they were transferred to S2 or G2.2 medium.

2.5.2 Morphological evaluation of embryo quality

Zygotes and cleaving embryos were observed and moved to fresh drops daily. The number and size of cells and degree of fragmentation were recorded. A grading system devised by Veeck (1990) was used to score embryo quality. This assessment of embryo quality relates to the symmetry of cleavage, degree of fragmentation and the rate of cleavage. Grade 1 represents an ideal morphology.

Grade1: Embryo with blastomeres of equal size, no cytoplasmic fragments
Grade 2: Embryo with blastomeres of equal size, minor cytoplasmic fragments or blebs. No more than 10% fragmentation of blastomeres
Grade 3: Embryo with blastomeres of distinctly unequal size, No more than 50% fragmentation of blastomeres.
Grade 4: Embryo with blastomeres of equal or unequal size, major cytoplasmic fragments (more than 50%) and numerous blebs.
Grade 5: Embryo with few or no recognizable blastomeres, major cytoplasmic fragmentation.
Grade 6: Embryos are non-viable, with lysed, contracted or dark blastomeres.

Computerized photographic images were collected each day until the embryos arrested or degenerated.

2.5.3 Fluorescent staining of chromatin

All oocytes that failed to mature or fertilize and all arrested embryos were stained with Hoechst 33258 (Sigma) to visualise chromatin to show the meiotic stage of the oocytes or the nuclear profile of blastomeres.

Stock Hoechst, 1mg/ml in sterile water for injections (B Braun Medical Ltd, Sheffield, UK) was prepared in advance and stored in the dark (wrapped in aluminium foil) at 4°C. For staining, a 1/100 dilution was prepared in phosphate buffered saline (PBS; Gibco). Oocytes/embryos were stained by incubation in 2ml prepared stain in a tissue culture dish at 37°C (on a warm stage) in the dark under aluminium foil for approximately 10 min and moved to fresh PBS at 37°C. Oocytes/embryos were viewed by placing in a small drop (~20µl) PBS on a slide with gentle compression under a coverslip supported by Vaseline pillars at the corners. The slides were viewed on a fluorescence microscope (Olympus B071) using a DAPI-filter. Fluorescent microscopic images were collected via video link and Image Pro-plus software (Media Cybernetics).

2.6 Chromosome spreading

Immature oocytes donated by patients undergoing ICSI treatment were cultured with colcemid (Sigma) at a final concentration of 10µg/ml in EBSS + 0.5% hSA in a prepared tissue culture dish (Falcon, Fahrenheit) having six 100µl drops of medium under ~7ml pre-equilibrated paraffin oil (Ovoil; Scandinavian IVF Science) Dishes were preincubated overnight at 37°C with 5% CO_2 in air before use, and oocytes were incubated for 4-6 hrs at 37°C, under 5% CO_2 in air before fixing and spreading.

Chromosome spreading was performed according to a modification of the air-drying technique (Tarkowski, 1966). Briefly, oocytes were placed in 1% w/v sodium citrate solution (BDH, Merck Ltd, Poole, Dorset, UK) at RT for ten minutes. Oocytes were then placed in a micro drop of the 1% w/v hypotonic sodium citrate medium in the middle of a grease free slide and gradual fixation was performed by adding a drop of freshly prepared fixative: ethanol (BDH): glacial acetic acid (Sigma) solution (v/v, 3:1). Blowing on the slide aided the air-drying procedure.

Fixed preparations were stained with 4% Giemsa (BDH) in 0.1M phosphate buffer (pH 7.2) in a coplin jar (BDH) for five minutes, then washed in 0.1M phosphate buffer (pH 7.2) alone. When chromosomes were observed, the slide was destained with ethanol and stained with ~20µl mounting medium containing 4'-6-diamidino-2-phenylindole at a concentration of 1.5µg/ml (DAPI; Vectorlabs, USA).

Fluorescence microscopy using a Zeiss Axioskop fluorescence microscope (Oberkochen, Germany) equipped with a DAPI (Zeiss 487909) selective filter set and a 50W mercury lamp was used to obtain the DAPI fluorescence. Images were taken using a cooled CCD camera (Photometrics) coupled with a Smartcapture image acquisition and analysis system (Vysis/Applied Imaging).

2.7 Immunocytochemistry

2.7.1 Fluorescent labelling of tubulin and chromatin

Tubulin and chromatin were identified in mouse and human oocytes. In vivo matured oocytes that had failed to fertilize were obtained from consenting patients undergoing IVF and used as control oocytes. Oocytes were fixed using a modification of the method of Battaglia *et al.* (1996a). Briefly, oocytes were fixed at RT for 45 min in PBS (Gibco) containing 16% w/v formaldehyde (TAAB Laboratories equipment Ltd, Berkshire, UK) and 0.01% Triton X-100 (Sigma). They were then washed in PBS containing 2% bovine serum albumin (BSA; Sigma) for at least 60 min.

The antibody employed to detect spindles was a monoclonal anti-α-tubulin antibody raised in mice and specific for human, monkey, murine and a variety of other species. It was supplied as solution in 0.01M PBS, pH 7.4, containing 1% BSA and 15mM sodium azide as a preservative, ready labelled with fluorescein isothiocyanate conjugate (FITC), isomer 1 (Sigma clone DM 1A). The antibody was applied at a concentration of 1:200 in PBS containing 0.1% Tween 20 (polyoxyethylenesorbitan monolaurate; Sigma) for 30 min at RT. After washing in PBS, the oocytes were mounted in PBS on a slide and stained with ~20µl mounting medium containing 4'-6-diamidino-2-phenylindole at a concentration of 1.5µg/ml (DAPI; Vectorlabs, USA) to localize chromatin; the coverslip was then placed on slide. The fixing and staining procedures were carried out at RT with the oocytes free in suspension.

Fluorescence microscopy using a Zeiss Axioskop fluorescence microscope (Oberkochen, Germany) equipped with fluorescein (Zeiss 487902) and DAPI (Zeiss 487909) selective filter sets and a 50W mercury lamp were used to obtain the FITC localization patterns and DAPI fluorescence simultaneously. Images were taken using a cooled CCD camera (Photometrics) coupled with a Smartcapture image acquisition and analysis system (Vysis/Applied Imaging). Paired and separate images were taken to examine the co-localization of tubulin and chromatin.

2.8 Image analysis

2.8.1 Set up and measurements

In this study, daily throughout culture, light microscopic images of all oocytes and embryos were routinely collected using an image analysis package (Image pro-plus, Media Cybernetics) linked via a video camera to an inverted microscope (Nikon) with Hoffman contrast optics. The store of images collected for each oocyte and patient was analysed to show whether any measured parameter correlated with the in vitro culture conditions employed and/or the subsequent development of the oocyte/embryo. Trial measurements were performed as a control to check the reliability of measuring (Table 7.1). For the trial, ten oocytes (from patients undergoing treatment in the CRM) were each measured ten times on the day of oocyte retrieval.

The image analysis package was used to measure various parameters of the oocyte or embryo, these were mean diameter of oocyte, mean diameter of oocyte + zona, mean zona thickness, and perivitelline space (PVS) (Figure A3, Appendix IV).

2.8.2 Oocyte diameter

The mean diameter of an oocyte was calculated by measuring the mean length of diameters as far as the oolemma at two-degree intervals and passing through the object's centroid.

2.8.3 Oocyte + zona diameter

This assessment was performed as for oocyte diameter, but extending the diameter measurement to the outer surface of the zona pellucida. It therefore included both the

oocyte and its zona pellucida, and incorporated differences in perivitelline space and zona thickness.

2.8.4 Zona pellucida thickness

The image analysis software generated a mean zona pellucida thickness measurement, after measuring the zona thickness at 2μm intervals.

2.8.5 Perivitelline space (PVS)

The image analysis software generated a measurement of the PVS by calculating the minimum and the maximum distance between the inner zona and the oolemma and calculating a mean PVS from this. This measurement could be assessed in one plane only and is therefore an estimate, which may vary depending upon the orientation of the oocyte.

2.9 Statistics

Scatter plots of oocyte yield (viable and atretic) according to patient age; weight, BMI or days since last menstrual period were assessed for correlation using regression analysis. P values < 0.05 were considered significant. Student's t-test was used to compare the numbers of viable oocytes from the two patient groups.

The proportions of GV and GVBD oocytes maturing or undergoing atresia in the presence or absence of EGF were compared using pxq contingency table with chi-squared test (χ^2) (Campbell, 1989).

The proportions of oocytes surviving, maturing or becoming atretic in groups exposed to different concentrations of FF-MAS were compared using pxq contingency table with χ^2 test. The extent of maturation in vitro of oocytes collected at the GV and GVBD stages and cultured with or without FF-MAS within both patient groups was tested for statistical significance using χ^2 test. The extent of oocyte maturation according to the amount of cumulus cover at recovery of oocytes from patients with PCO was tested for significance using pxq contingency table with χ^2 test.

The measurements for each individual oocyte over several days of assessment were compared, according to the treatment which the oocyte received and the outcome of attempted maturation and fertilization in vitro, to observe whether there were correlations between these measurements and the oocyte's subsequent development. In this way, normal and threshold values were identified for various features of oocyte development.

For oocytes retrieved from patients with PCO, diameters of oocytes with dense cumulus cover at collection were compared with oocytes with less or no cumulus cover using pxq contingency table with χ^2 test.

Within both patient groups the following tests were performed: oocytes diameters on day 0 were compared according to the outcome of in vitro culture (mature, immature, atretic) and tested for statistical significance using the Kruskall-Wallis test (Campbell, 1989). For each patient group, oocyte diameters were compared between day of oocyte retrieval and day 0 of oocytes that became atretic, within each culture condition using the Mann-Whitney U-test (Campbell, 1989). Oocyte growth during culture (from day of oocyte retrieval to day 0) for those oocytes that matured was tested for statistical significance using the non-parametric sign test (Campbell, 1989), according to the conditions of culture to which oocytes were exposed. For the difference in growth between oocytes maturing in 30μg/ml MAS and in control, a two-sample t-test was performed.

The difference in mean diameter of oocytes + zona between the day of oocyte retrieval and day 0 within each culture group was tested for statistical significance using the Mann Whitney U-test. A pxq contingency table with χ^2 test was performed on oocyte + zona data on day of oocyte retrieval and on day 0 for those measuring 140-151μm and 152-175μm and the ability of the oocytes to mature in vitro. The same test was also performed on oocyte + zona data on day of oocyte retrieval and on day 0 for those measuring 140-157μm and 158-175μm. A Mann Whitney U-test was performed on the zona pellucida thickness of oocytes on day+1 of those that fertilized and those that failed to fertilize/became atretic for both patient groups.

A one-tailed T-test was performed on oocyte diameters on day of collection from the two patient groups. A Mann Whitney U-test was performed on oocyte + zona diameters on the day of collection from the two patients groups.

Additionally for oocytes donated by patients undergoing ICSI, non-parametric statistical analyses (Mann Whitney U tests) were applied to determine if there were any significant differences in oocyte diameter between oocytes that matured within 23-24 hr and those that matured within 40-48 hr. This was performed for oocytes within each culture group, and using pooled data (all culture groups combined). A Kruskall Wallis test was also performed on oocytes diameters of all those maturing within 23-24 hr within the different culture groups, and on all those maturing within 46-48 hr within the different culture groups.

Chapter 3

Patient data

3.1 Introduction

Immature oocytes were retrieved from two patient groups: unstimulated patients having PCO undergoing treatments which required a laparoscopy; and patients undergoing ICSI treatment, which included ovarian stimulation according to a standard 'long' down-regulation protocol. The study was approved by Coventry Research Ethics Committee and licensed by the Human Fertilisation and Embryology Authority.

Oocyte function is known to be related closely to the environment of the follicle, and the health of the somatic cells that surround and nourish the oocyte. The origin of the oocytes used to study oocyte maturation may therefore influence their subsequent viability and developmental competence. This chapter presents basic information about the patients who donated oocytes to this study, including their ages, aetiology of infertility, outcome of treatment and other parameters that may have had a bearing upon the quality of their oocytes.

For the patients with PCO, this information has been related to the oocyte yield of the unstimulated oocyte collection procedure. Since patients undergoing ICSI treatment received ovarian stimulation, predictive information would not be expected to arise from oocyte yield and this approach has not been taken.

3.2 PCO patients

3.2.1 Clinical details of patients

The clinical details of patients with PCO are presented in tables 3.1 and 3.2 according to whether their oocytes were used in the EGF or MAS series of experiments respectively.

Table 3.1 Clinical details of patients with polycystic ovaries (PCO) who donated immature oocytes for culture with or without EGF.

Patients (n)	8
Patient age (years)[c]	31.8 ± 5.8 (25-37)
Menstruation	
Regular (28-35d)	0
Oligomenorrhoeic	6 (days since LMP: 37.3 ± 14.9, range 16-55)
Amenorrhoeic (2°)[a]	2 (days since LMP: 304 ± 257.4, range 122-486)
Weight (kg)	72.9 ± 19.3 (51-96)
Body mass index (BMI)[b]	30.5 ± 7.6 (25-45)
No of oocytes collected[c]	
Live	5.8 ± 5.9 (1-19)
Atretic	5.1 ± 4.7 (0-11)

[a] 2°Amenorrhoea is defined as no menstruation for >3 months.

[b] BMI calculated as body weight (kg) divided by height (M^2).

[c] Data are mean ± SD, with range in parentheses.

Table 3.2 Clinical details of patients with polycystic ovaries (PCO) who donated immature oocytes for culture with or without FF-MAS.

Patients (n)	19	
Patient age (years) [c]	27.9 ± 3.8	(22-35)
Menstruation		
Regular (28-35d)	3	(days since LMP: 21.3 ± 5.8, range = 18-28)
Oligomenorrhoeic	10	(days since LMP: 25.9 ± 26.8, range = 3-96)
Amenorrhoeic (2°) [a]	5	(days since LMP: 192.2 ± 33.8, range =154-241)
Data not recorded	1	
Weight (kg)	75.3 ± 19.9	(44-115)
Body mass index (BMI) [b]	30.4 ± 7.0	(15.5-44)
No of oocytes collected [c]		
Live	6.7 ± 4.0	(1-19)
Atretic	5.1 ± 4.3	(0-14)

[a] 2°Amenorrhoea is defined as no menstruation for >3 months.

[b] BMI calculated as body weight (kg) divided by height (M^2).

[c] Data are mean ± SD, with range in parentheses.

A comparison of tables 3.1 and 3.2 revealed no significant differences between the groups in terms of patient age, weight, BMI or number of live or atretic oocytes collected. The PCO patient group has therefore been presented as a cohort of 27 patients for the subsequent analyses in this chapter.

23% (5/26) patients became pregnant as a result of surgery; one patient was undergoing surgery to decrease hirsuitism and was sterilized after the oocyte collection.

3.2.2 Relationship between age and number of oocytes collected.

Figure 3.1 shows scatter plots of the numbers of viable and atretic oocytes collected from unstimulated PCO ovaries according to the age of the patient. The figure shows no significant correlation between age and number of oocytes collected, whether viable or atretic.

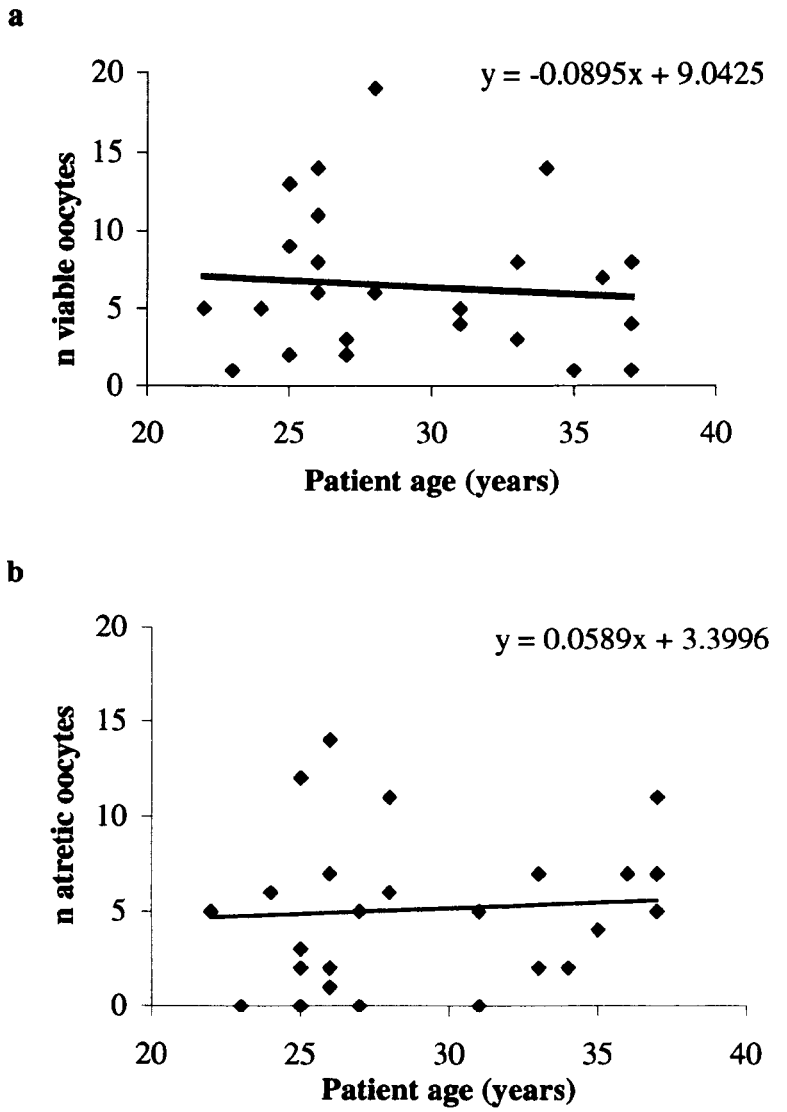
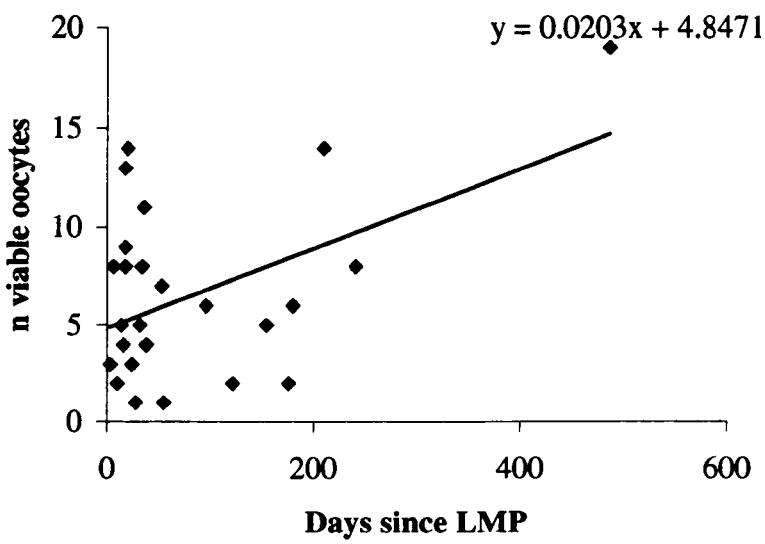


Figure 3.1 Scatter plots of the number of (a) viable and (b) atretic oocytes retrieved from unstimulated polycystic ovaries according to the age of the patient.
No significant correlations were present **(a)** r = 0.092 **(b)** r = 0.064.

3.2.3 Relationship between days since last menstrual period (LMP) and the number of viable and atretic oocytes collected.

Figure 3.2 presents a scatter plot of the numbers of viable and atretic oocytes retrieved from unstimulated patients with PCO according to the time that had elapsed since their last menstrual period. The apparent positive correlations between these parameters in terms of viable and atretic oocytes were not significant and may have occurred because of a single outlying observation for each, skewing the distributions.

a



b

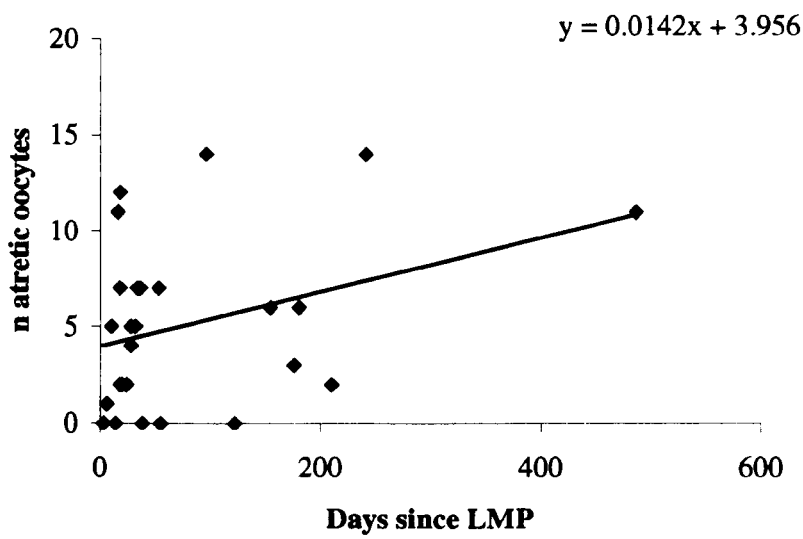


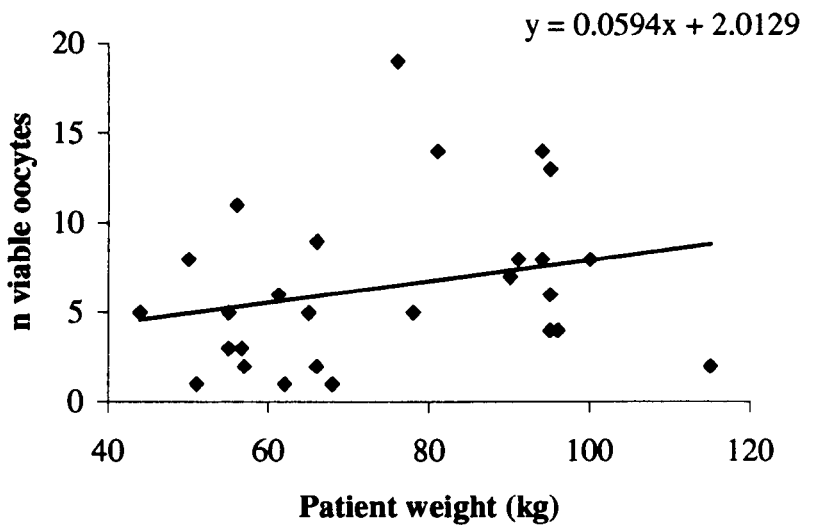
Figure 3.2 Scatter plots of the number of (a) viable and (b) atretic oocytes according to the number of days since the last menstrual period (LMP).
No significant correlations **(a)** r = 0.471 **(b)** r = 0.359.

3.2.4 Relationship between patient weight and the number of viable and atretic oocytes collected.

Extremes of weight are known to affect fertility, and a tendency towards obesity may occur in women with PCO. Fertility may be enhanced in obese women with PCOS by a loss of weight (Jansen, 1995). Figure 3.3 presents the scatter plots of the numbers of viable/atretic oocytes retrieved from women with PCO in relation to their weight in kg.

Although this study group included a range of obese and non-obese women, no significant associations between weight and viable or atretic oocyte yield were observed.

a



b

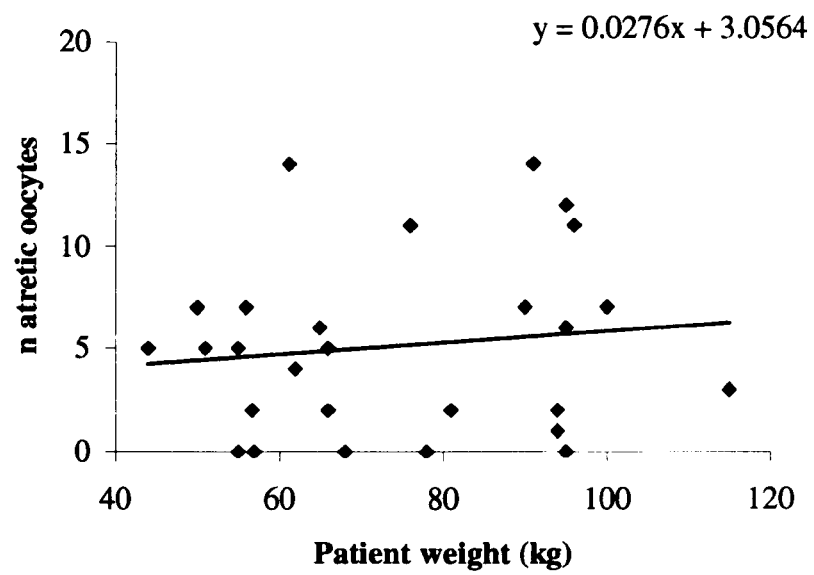


Figure 3.3 Scatter plots of the number of (a) viable oocytes and (b) atretic oocytes retrieved according to the weight of patients with PCO.
No significant correlations, **(a)** r = 0.252 **(b)** 0.124

3.2.5 Relationship between patient body mass index (BMI) and the number of viable and atretic oocytes collected.

BMI is an index of obesity; it is calculated as body weight (kg) divided by height (M^2). A BMI <20 kg/m^2 is considered underweight and >30 kg/m^2 is considered obese (Balen *et al.*, 1999). Figure 3.4 presents the scatter plots of the numbers of viable and atretic oocytes retrieved from women with PCO in relation to their BMI. No significant associations between BMI and viable or atretic oocyte yield were observed.

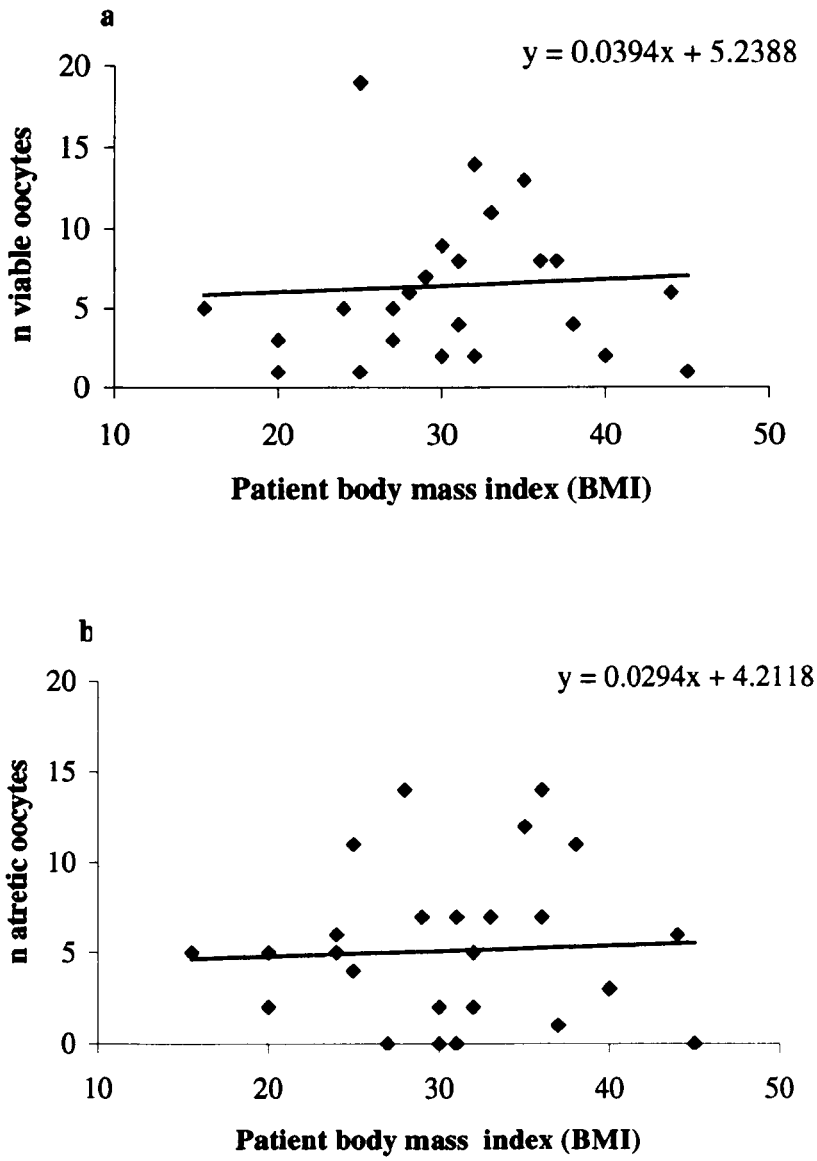


Figure 3.4 Scatter plots of the number of (a) viable oocytes and (b) atretic oocytes retrieved according to the BMI of PCO patients.
No significant correlations, **(a)** r = 0.061 **(b)** 0.048.

3.3 ICSI patients

3.3.1 Clinical details of patients

Table 3.3 presents the clinical details of all the patients included in this study who were undergoing ICSI treatment. They were recruited between September 1998 and April 2001, during which time ICSI procedures at the CRM remained unchanged. The data in table 3.3 indicate rates of successful pregnancy that are consistent with the IVF/ICSI programme of the CRM over this period. A total of six patients had partners diagnosed with azoospermia. Of these, three patients had epididymal sperm used and three had testicular sperm used for the therapeutic ICSI procedure, which did not affect their success in terms of oocyte fertilization and pregnancy.

Table 3.3 Clinical details of patients undergoing intracytoplasmic sperm injection (ICSI) treatment who donated immature oocytes for culture with or without FF-MAS, chromosome assessment or for culture with or without FF-MAS for spindle analysis.

Patients (n)	57	
Patient age (years)[a]	32.9 ± 3.7	(27-40)
No. of previous ICSI attempts[a]	0.8 ±. 0.9	(0-3)
Total no. of oocytes collected[a]	15.2± 6.2	(5-28)
Fertilization rate (2PN)[b]	63.5%	
Ongoing pregnancy rate	29.6%	(16/54[c, d]) 15 delivered
Pregnancy losses	1[e]	
No. of cleaving embryos frozen	2.2 ± 2.4	
No. of 2PN frozen	0.9 ± 2.5	
No. of immature oocytes donated to study	2.2 ± 1.5	(1- 6)

[a] Data are mean ± SD, with range in parentheses.

[b] Oocytes with 2 PN/ oocytes injected

[c] For one cycle, all embryos frozen

[d] Two cycles resulted in OHSS, therefore no embryo transfer

[e] Biochemical pregnancy

Table 3.4 presents the principle identifiable causes of infertility of the patients who donated immature oocytes via the ICSI programme. While these pertain to the results of treatment presented in table 3.3, they are less relevant for the subsequent experimental work on immature oocytes, in which fertile donor sperm was used to promote consistency of interpretation.

Table 3.4 Aetiology of infertility for patients (n=57) undergoing ICSI treatment who donated immature oocytes for this study.

Aetiology of infertility	No. of patients	Cause	No. of patients	Mean no. of years of involuntary infertility	Range
Primary (1°)	36	♂ **factor:**		5.0 ± 2.8	1-12
		Teratozoospermia	20		
		Azoospermia	3		
		Oligozoospermia	3		
		♀ **factor:**			
		Ovarian	1		
		Tubal	4		
		♂ **&** ♀ **factor:**	2[a,b]		
		Failed/low fert:	3		
Secondary (2°)	21	♂ **factor:**		4.0 ± 2.4	1-12
		Teratozoospermia	7		
		Azoospermia	3		
		Oligozoospermia	5		
		Failed vasectomy reversal	1		
		♀ **factor:**	2		
		Ovarian	1		
		Tubal	1		
		♂ **&** ♀ **factor:**	1[a]		
		Failed/low fert:	0		

[a] Anovulatory and teratozoospermia

[b] Endometriosis and teratozoospermia

Definition of terms (WHO, 1999):

Teratozoospermia-Reduced levels of normally shaped sperm (<15% normal)

Azoospermia-Total absence of sperm

Oligozoospermia-Reduced sperm numbers (<20 x 10^6/ml)

3.4 Discussion

The patients having polycystic ovaries, who donated oocytes during gynaecology operations comprised one group, regardless of which part of the study their oocytes were used in, as shown in tables 3.1 and 3.2.

The mean number of viable oocytes collected from this group of patients with PCO was 6.4 ± 4.6. This was lower than the means of 13.8 and 13.1 obtained from anovulatory and ovulatory PCO patients (collected in the follicular phase) respectively by Trounson *et al.* (1994), but more comparable to the average of 8.1 per patient reported by Coskun *et al.* (1998). Coskun *et al.* (1998) recovered immature oocytes from 17 patients at risk for ovarian hyperstimulation syndrome, ten of the patients had polycystic ovaries. Immature oocytes were recovered transvaginally with reduced aspiration pressure, one day after the last hMG injection, hCG was not administered. Russell *et al.* (1997) retrieved a similar figure of approximately 11 oocytes from unstimulated patients whose endometrial priming started in either the early or midfollicular phase.

Both Coskun *et al.* (1997) and Russell *et al.* (1997) collected the oocytes transvaginally under ultrasound guidance; therefore all of the visible antral follicles were aspirated. Trounson *et al.* (1994) also collected the oocytes transvaginally under ultrasound guidance, except in 19 % (8/42) of patients, for whom laparoscopic oocyte retrieval was performed, due to the difficult puncture of small follicles as a result of ovary mobility. In comparison, the oocytes collected from PCO patients in this study were all collected laparoscopically; therefore follicles were rarely clearly visible prior to aspiration, the patients had received no gonadotrophin priming and the laparoscopy was scheduled irrespective of the menstrual cycle. This may explain both the reduced number of oocytes retrieved and the collection of atretic oocytes (mean number of 5.1 ± 4.3 atretic oocytes per PCO patient). In a study of immature oocyte retrieval in 37 patients with varying diagnoses including tubal disease, endometriosis, anovulation, male factor and unexplained, Russell *et al.* (1997) reported a mean of 2.1 atretic oocytes per patient when oocyte retrieval was scheduled between day 9 and day 12.

The appearance of the atretic oocytes in the present study was characteristically dark indicating that they had been atretic for some time, rather than damaged during the aspiration procedure.

The small follicles associated with polycystic ovaries are neither atrophic nor apoptotic (Homburg *et al.,* 1996) but may persist longer than normal at this stage. They became arrested in the small antral stage following apparently normal development up to a size of 6-8mm, the size at which dominant follicle selection would normally take place (Fauser, 1994). The effects which such persistent follicles may have upon oocyte viability is uncertain since they are highly responsive to FSH stimulation and may result in viable mature oocytes in assisted conception procedures.

Due to the small size of follicles present in polycystic ovaries, the technique of oocyte retrieval is one of the pivotal factors for success (Cha *et al.,* 2000). Using an US-guided transvaginal oocyte aspiration technique, either 10-13 days after spontaneous menses in those PCOS patients with some menstrual function or 10-13 days after withdrawal bleeding induced by IM injection of progestin, Cha *et al.* (2000) reported the recovery of 13.6 ± 7.5 oocytes per patient, with a range of 3-34. The induction of a withdrawal bleed, which effectively "restarts" follicle development by promoting endogenous FSH secretion, may have assisted in the achievement of a high yield of oocytes.

The presence of small antral follicles responsive to FSH in the luteal phase is thought to determine the number of recruitable oocytes in the following menstrual cycle (Gougeon and Testart, 1990), therefore an increase in FSH levels in the late luteal phase would possibly allow more follicles to continue their growth in the following menstrual cycle (Suikkari *et al.,* 2000). Suikkari *et al* (2000) reported the mean recovery of 11.2 viable oocytes from women with regular menstrual cycles and a mean of 10 viable oocytes from women with PCO and irregular cycles, after low-dose FSH priming of follicles starting in the late luteal phase. The women with PCO in this study received no gonadotrophin priming and so the pool of follicles for aspiration may have been smaller.

In contrast to the reports cited above, preliminary studies of the pretreatment of women with FSH early in the follicular phase showed no difference in the recovery rate of oocytes (Trounson *et al.,* 1998; Mikkelsen *et al.,* 1999), although improved maturation was observed (Wynn *et al.,* 1998).

The number of oocytes available to be collected would be expected to reduce as the ovarian reserve declines with advancing age, as evidenced by rising basal FSH levels (Cha and Chian 1991 Cha *et al.,* 1991; Whitacre *et al.,* 1998) and the symptoms of PCO may ameliorate with increasing age. However, Figure 3.1 demonstrates no significant correlation between age and the number of oocytes collected, whether viable or atretic. The underlying condition of PCO, where many antral follicles persist may account for the observed numbers of oocytes failing to decrease significantly with increasing age, as might otherwise be expected.

Barnes *et al.* (1996) reported the retrieval of a larger number of oocytes from irregularly cycling and anovulatory patients compared to regularly cycling women (16.5 and 4.9 respectively), which related to the relative ease of aspiration from the peripherally located antral follicles in PCO. Among patients with PCOS, Cha *et al.* (2000) retrieved a similar number of oocytes from women with oligomenorrhea and amenorrhea. Almahbobi *et al.* (1996) proposed that ovulatory PCO is an intermediate form of the syndrome, which may lead, when expressed severely to anovulatory PCO. In this study, the time elapsed since LMP did not show any significant correlations with the numbers of viable or atretic oocytes retrieved. It had been expected that the time elapsed since the LMP may correlate with an increasing number of atretic oocytes as a result of follicle aging, however, this supposition was not confirmed.

Obese women with PCOS hypersecrete insulin, which stimulates ovarian secretion of androgens, and is associated with hirsuitism, menstrual disturbance and infertility (Balen *et al.,* 1999). It is known that weight loss can lead to regular menstrual cycles and spontaneous pregnancies by producing a more favourable ovarian environment for follicle growth through a lower insulin and insulin-like growth factor (IGF)-I concentration (Franks, 1989; Pasquali *et al.,* 1989; Kiddy *et al.,* 1990, 1992 and Elting

et al., 2000). In this study, no significant associations between weight or BMI and viable or atretic oocyte yield were observed (Figure 3.3 and 3.4 respectively).

In conclusion, no specific characteristics of the PCO patients in this study were predictive of the numbers of oocytes retrieved; specifically patient age, time since the LMP, weight and BMI did not show significant correlations with either viable or non-viable oocytes. This may be related to the pathological "PCO" ovary, where large numbers of antral follicles are present at any age, potentially tending to equalize any differences in accessible follicle numbers due to underlying age or other factors related to the ovarian reserve.

The patients undergoing ICSI treatment and donating immature oocytes appeared representative of infertility patients, who would normally undergo this treatment having a predominance of male factor indications. The mean age of patients in this study (32.9 ± 3.7) is similar to the mean age of patients attending the CRM for infertility treatment, which is ~34 years. The mean total number of oocytes collected per patient (15.2 ± 6.2) appeared generally higher than the mean number of oocytes normally collected for infertility patients undergoing oocyte collection at the CRM, which is ~10 oocytes. Patients in this group had clearly responded well to stimulation, as might be anticipated from the relatively low incidence of ♀ factor indications for treatment, and also from the observation that their oocyte cohort included one or more immature oocytes. These would be expected to arise from smaller or less mature follicles and might suggest a tendency towards OHSS or PCO amongst some of these patients. The pregnancy rate shown in table 3.3 was similar to the ICSI pregnancy rate of infertility patients at the CRM over the same period. The number of immature oocytes donated by patients undergoing ICSI ranged from 1-6 and might indicate that a proportion of follicles failed to develop fully in response to the ovarian stimulation.

The exogenous gonadotrophic drugs used to stimulate the growth of several follicles concurrently may compromise oocyte development in comparison with natural cycles (Enien *et al.*, 1995). The immature oocytes obtained in this study after follicular stimulation have been exposed to supraphysiological concentrations of gonadotrophins in vivo yet still failed to mature, they may therefore be of inherently

reduced quality or the immature oocytes may have been developing normally but retrieved from small follicles with reduced gonadotrophin sensitivity. Alternatively, they may have arisen from otherwise normal follicles, which did not receive the same hormonal stimulus as others, perhaps due to a limited blood supply. However, such oocytes have been previously shown, in certain cases to have the potential for full embryonic developmental (Cha and Chian, 1998).

No patient undergoing ICSI who donated oocytes to this study had immature oocytes retrieved only. This is a rare occurrence with few previous case reports (Hartshorne *et al.*, 1999). Patients donating oocytes in this study had a mean of 11.4 ± 5.6 mature oocytes collected that could be used for their treatment, and their donation of immature oocytes to this study had no effect upon their treatment.

While the collection of oocytes from patients undergoing infertility treatment or suffering pathological conditions is not ideal for research, the extremely limited availability of human oocytes for study required such an approach to be taken. Fresh, in vivo matured oocytes are reserved for patients' own use, and are therefore not accessible as control material, without interfering with the patient's chances of a successful outcome. It is hoped that if IVM can be optimized, then further understanding of the processes involved in oocyte and embryo development could be gained from the increased availability of fertile oocytes that would arise as a result. Additional sources of such immature oocytes might include patients undergoing laparoscopic sterilization, caesarian section, or ovariectomy. However, these would also have concerns, such as the likelihood of prior steroidal contraception, an acyclic endocrine environment, or advanced age associated with a low ovarian reserve or malignancy potential respectively. There therefore remains no optimal source of oocytes, however, a through understanding of the patient populations from which starting material is sourced is essential in interpretation of experimental results.

Chapter 4

Addition of EGF to culture of oocytes from PCO patients

4.1 Introduction

A number of peptide growth factors have been implicated as ovarian regulators of follicular development and steroidogenesis. These factors include, for example, epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), fibroblast growth factor (FGF), insulin-like growth factor-type I (IGF-I), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGFβ) (Yeh *et al.*, 1993).

EGF is present in human follicular fluid at concentrations ranging between 0.60-2.42ng/ml (Westergaard *et al.*, 1990). Serum concentrations of EGF range between 0.7 and 2.0ng/ml in humans (Hofmann *et al.*, 1990; Westergaard and Andersen, 1989; Westergaard *et al.*, 1990).

In 1990, Westergaard *et al.* studied EGF concentrations in small antral follicles (<6mm in diameter) of pregnant women and reported concentrations of EGF in the follicular fluid of these follicles to be several times higher than those in serum. In addition, a significant decline in the levels of intrafollicular EGF from 4.7 to 1.4nmol/l was observed with increasing follicle size from 1-6mm. This decrease in intrafollicular EGF seen with follicular growth appears to continue until the preovulatory stage (Westergaard *et al.*, 1990). In a previous study of EGF concentrations in follicular fluid aspirated from preovulatory follicles (>14mm diameter) by Westergaard and Yding-Andersen (1989), the EGF concentration in follicular fluid ranged between 0.1-0.4nmol/l, corresponding to approximately 50% of that in serum. This led Westergaard *et al.* (1990) to conclude that intrafollicular EGF is specific to small antral follicles and may be synthesized by the granulosa and/or theca cell layers of these follicles.

Data obtained in the mouse model by Almahbobi *et al.* (1995) also suggested that in normal conditions, the decrease in the concentrations of EGF/TGF-α is a necessity for late follicular maturation.

EGF has been implicated in the condition of polycystic ovaries (PCO), due to its potent inhibition of FSH-induced aromatase activity (Franks *et al.*, 1988; Mason *et al.*, 1990). Franks *et al.* (1987, 1988) have suggested that EGF in small follicles may be responsible for a lack of follicular responsiveness to FSH in women with polycystic ovary syndrome. These women exhibit a significantly higher level of EGF in follicular fluid obtained from Graafian follicles than women with normal ovaries. It is possible that PCO may be caused by a disruption in the regulatory mechanisms of EGF/TGF-α production and activity, resulting in a reduction in oestradiol concentration and an accumulation of androgens. The persistence of EGF activity, in conjunction with the subsequent lack of oestradiol, would inhibit LH receptor formation on granulosa cells. As a result, growth and development of the dominant follicle would be inhibited, leading to anovulation (Almahbobi *et al.*, 1995). Most of the follicles in patients with PCO are not atretic (Almahbobi *et al.*, 1996) and furthermore follicular atresia, at least in its early stages, does not appear to reduce the developmental competence of human oocytes (Barnes *et al.*, 1996).

Interestingly, and in contrast to the above data on the potential aetiology of PCO, EGF plays a positive role in various mammalian culture systems supporting oocyte maturation (Goud *et al.*, 1998). The promotion of oocyte nuclear maturation by EGF has been demonstrated in humans (Das *et al.*, 1991; Gómez *et al.*, 1993a,b), mice (Das *et al.*, 1991; Downs *et al.*, 1989; De La Fuente *et al.*, 1999), cattle (Kobayashi *et al.*, 1994; Lorenzo *et al.*, 1994; Lonergan *et al.*, 1996; Park *et al;* 1997; Reiger *et al.*, 1998), and pigs (Singh *et al.*, 1993; Ding and Foxcroft 1994; Grupen *et al.*, 1997). In addition, EGF has also been shown to promote oocyte cytoplasmic maturation in the same species (humans: Goud *et al.*, 1998, mice: Das *et al.*, 1991; De La Fuente *et al.*, 1999; cattle: Kobayashi *et al.*, 1994; Lonergan *et al.*, 1996; Park *et al;* 1997; Reiger *et al.*, 1998; pigs: Ding and Foxcroft 1994; Singh *et al.*, 1997; Wang and Niwa, 1995; Grupen *et al.*, 1997; Abeydeera *et al.*, 1998).

EGF is a potent stimulator of oocyte maturation in the mouse (Dekel and Sherizly, 1985; Pellicer *et al.*, 1989 and Downs 1989), and in combination with gonadotrophins, EGF promotes nuclear maturation in oocytes obtained from intact preantral follicles cultured to the antral follicle stage (Boland and Gosden, 1994;

Smitz *et al.*, 1998). Positive effects were observed at ≥5ng/ml. EGF also induces nuclear maturation in human immature unstimulated oocytes (Das *et al.*, 1991; Pellicer *et al.*, 1992; Gomez *et al.*, 1993b).

In 1998, Goud *et al.* studied the role of cumulus cells and EGF (2ng/ml) in the culture medium on IVM of human GV oocytes obtained after gonadotrophin stimulation for ICSI. EGF was shown to have a direct action on both cumulus-intact and cumulus denuded oocytes during IVM. Furthermore, retention of the attached cumulus cells helped to achieve higher rates of cytoplasmic maturation indicated by the higher cleavage rates after fertilization. More recently, EGF (2ng/ml) in combination with FSH present during the first 24 hours of culture of human mature oocytes has been shown to augment the implantation rates of the resulting embryos, even though the positive effects were not evident during pre-implantation development (Yding-Anderson *et al.*, 1999).

Limited data are available on the role of EGF in IVM of human oocytes in the presence and absence of cumulus cells and in terms of its effects on embryonic competence. The culture system used for IVM is one of the most important factors in regulating the proportion and quality of oocytes maturing in vitro (Goud *et al.*, 1998). As yet, the optimal culture conditions and culture media components for human IVM have not been determined. This would be essential before introduction to clinical use. New developments in human assisted conception are sometimes based upon experience with other mammalian species and this approach is favoured in the work up of new techniques by the regulatory authorities. Therefore it was decided to assess the effects of EGF in a human IVM culture system, using similar conditions to those previously used in animal systems.

The effect of supplementation of the IVM culture medium with EGF was investigated in human immature oocytes obtained from unstimulated ovaries from women with PCO. The EGF concentrations used (0.1-10ng/ml) were similar to those shown to be effective in previous studies (Das *et al.*, 1991; Gomez *et al.*, 1993a,b; Goud *et al.*, 1998). The nuclear and cytoplasmic maturation of the cultured oocytes was judged

from their capability to undergo GVBD, polar body extrusion, fertilization and early cleavage after ICSI.

4.2 Aims

To collect immature oocytes from patients without recent history of ovarian stimulation or fertility drugs, having polycystic ovaries coincident with diagnostic laparoscopy or laser drilling.

To culture immature oocytes, in order to assess the effects of EGF upon oocyte maturation in vitro.

To inseminate in vitro matured (metaphase II) oocytes with known fertile donor sperm using ICSI in order to assess any effects of EGF on fertilization, cleavage and embryo development.

4.3 Patients and oocytes

4.3.1 Patients

For this study, a total of 46 viable immature oocytes were collected from eight PCO patients with unstimulated ovaries. Clinical details of the patients are presented in Chapter 3, Table 3.1.

4.3.2 Oocytes

The viable oocytes collected were randomly allocated to four groups: 0 (control), 0.1, 1.0 and 10ng/ml EGF for a period of 48 hrs. Oocytes were considered mature if there was evidence of a polar body visible by Hoffman microscopy. All mature oocytes were subjected to ICSI with donor sperm as described in Chapter 2, section 2.4.3.

4.4 Effect of EGF on survival and maturation

Figure 4.1 shows the results of EGF exposure in terms of oocyte survival and maturation, according to the concentration of EGF present in the culture medium used for IVM. The apparent increase in survival with increasing EGF concentration did not reach statistical significance, 73% survival in vehicle ranging to 89% in 10ng/ml EGF. The proportion of surviving oocytes which matured, was ~50% in all the EGF culture concentrations.

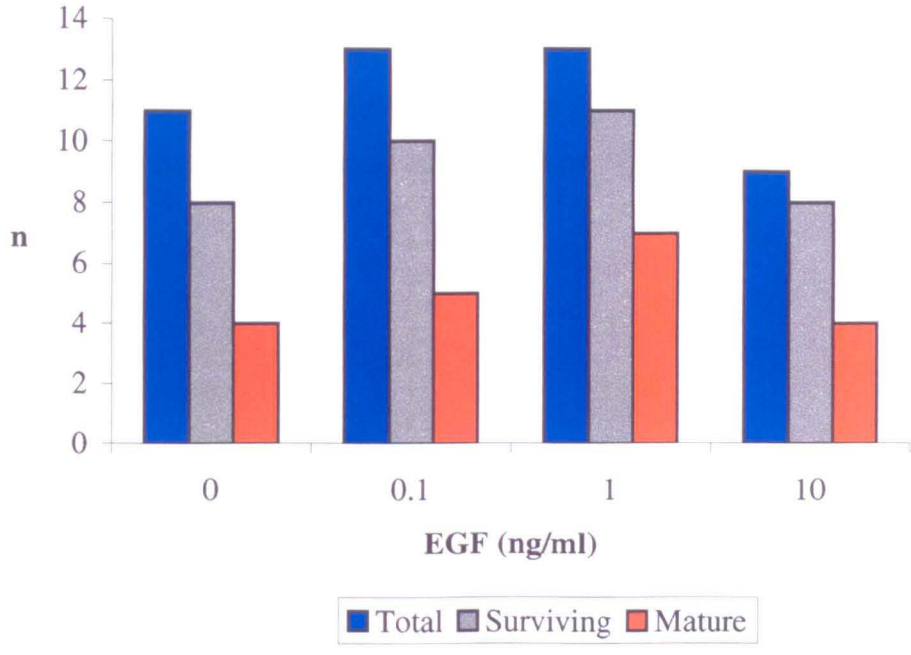


Figure 4.1 In-vitro maturation of immature oocytes (n=46) collected from patients (n=8) with polycystic ovaries and cultured with or without epidermal growth factor (EGF).

The oocytes collected were at the GV and GVBD stages in equal proportions. Figure 4.2 shows the maturation in vitro of oocytes collected at the GV and GVBD stages. It was notable that in the absence of EGF, none of the five GV oocytes matured to MII, whereas in the presence of EGF a total of nine of the 19 GV oocytes matured to MII. This represented a significant difference ($0.025 < p < 0.05$). However, the proportion of maturing GV oocytes was unrelated to the concentration of EGF. There was no significant difference between the proportions of GVBD oocytes maturing to MII with or without EGF. All oocytes that matured produced polar bodies on the second day of IVM culture (40-48 hr). Once the oocytes had matured, there were no visible differences among the groups, for example in the size of the oocyte and the size of the polar body between the different culture groups.

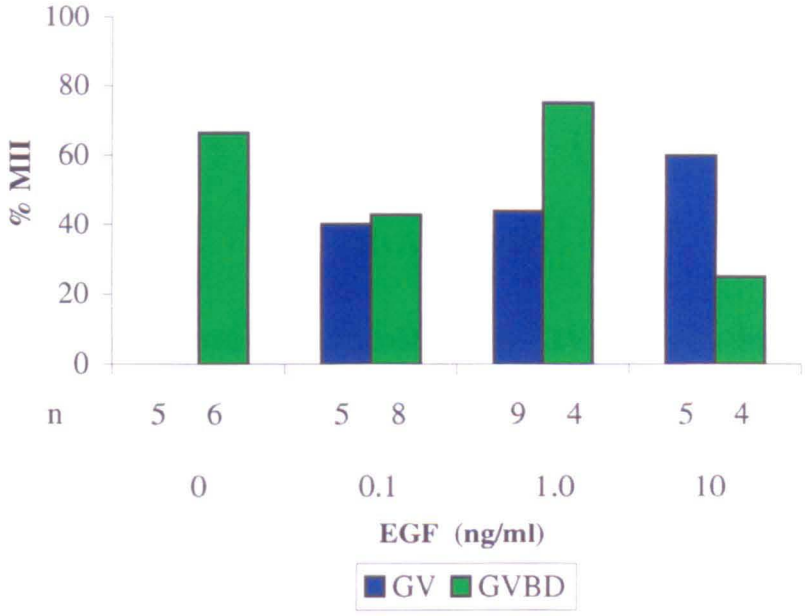


Figure 4.2 Maturation of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) oocytes from patients with polycystic ovaries (PCO) in the presence or absence of epidermal growth factor (EGF).
n= number of oocytes in each group.

Figure 4.3 shows the incidence of atresia occurring in vitro after the collection of apparently viable oocytes. There was no significant difference between atresia levels with or without EGF, or between GV and GVBD oocytes in each culture condition (with or without EGF).

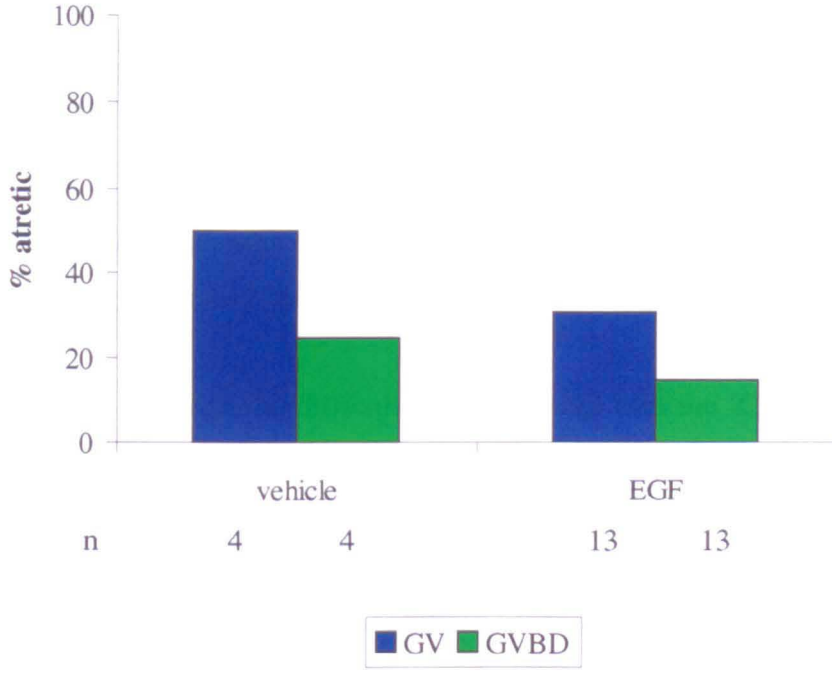


Figure 4.3 Proportions of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) oocytes becoming atretic in vitro after collection from patients with polycystic ovaries (PCO) and cultured in the presence or absence of EGF.
n= number of oocytes in each group.

4.5 Effects of EGF on fertilization and embryo development

Table 4.4 presents the proportions of maturing oocytes fertilized by ICSI, resulting in formation of two pronuclei and those subsequently cleaving in vitro. Statistical tests were not performed in view of the small amount of data available for analysis.

Table 4.4 Fertilization and cleavage rates of in-vitro matured oocytes arising from polycystic ovaries (PCO) patients.

	Epidermal growth factor (ng/ml)			
	0	0.1	1.0	10
Fertilization*	3/3	2/5	2/5	1/4
Cleavage**	2/3	2/2	1/2	1/1

* 2pn oocytes / oocytes surviving ICSI

** Cleaving embryos / 2pn oocytes

There were no particular difficulties encountered with the ICSI procedure. 2 cases of abnormal fertilization were observed at the highest EGF concentrations (1.0 and 10ng/ml): fragmented pronuclei were seen in an oocyte matured in 1.0ng/ml EGF and 3 pronuclei were seen in an oocyte matured in 10ng/ml EGF. Only one polar body was seen in the case of triploid fertilization, so it is likely that retention of the second polar body occurred during oocyte meiotic maturation, leading to the formation of two maternal pronuclei and one paternal pronucleus resulting in a digynic embryo. The most advanced embryo observed reached 6 cells (0.1ng/ml EGF).

Failure of cytokinesis was observed in two embryos (oocytes matured in vehicle and 0.1ng/ml EGF). One 2-cell embryo had five nuclei, all five nuclei were in the larger cell and no chromatin was seen in the smaller cell and a 3-cell embryo contained four nuclei in one cell indicating nuclei division without cell division (karyokinesis) and one nucleus each in the other two cells. The other embryos each had one nucleus per cell as would be expected for normal development.

It is usual for some oocytes to become damaged during the ICSI procedure itself, normally amounting to approximately 9% (Tarlatzis and Bili, 1998), unless the

oocytes are particularly fragile. In this series, 25% of oocytes that matured without EGF (control) underwent atresia compared to 13% in the combined EGF culture groups. This did not represent a significant difference.

4.6 Effect of cumulus cells on oocyte development

Figure 4.4 shows the extent of oocyte maturation according to the amount of cumulus cell cover at oocyte recovery. There were no significant differences in the proportions of oocytes maturing to MII, or becoming atretic, according to whether they were or were not surrounded by cumulus cells. The small numbers of oocytes available may have limited interpretation, however, as there were tendencies towards improved maturation and reduced atresia in the presence of cumulus cells.

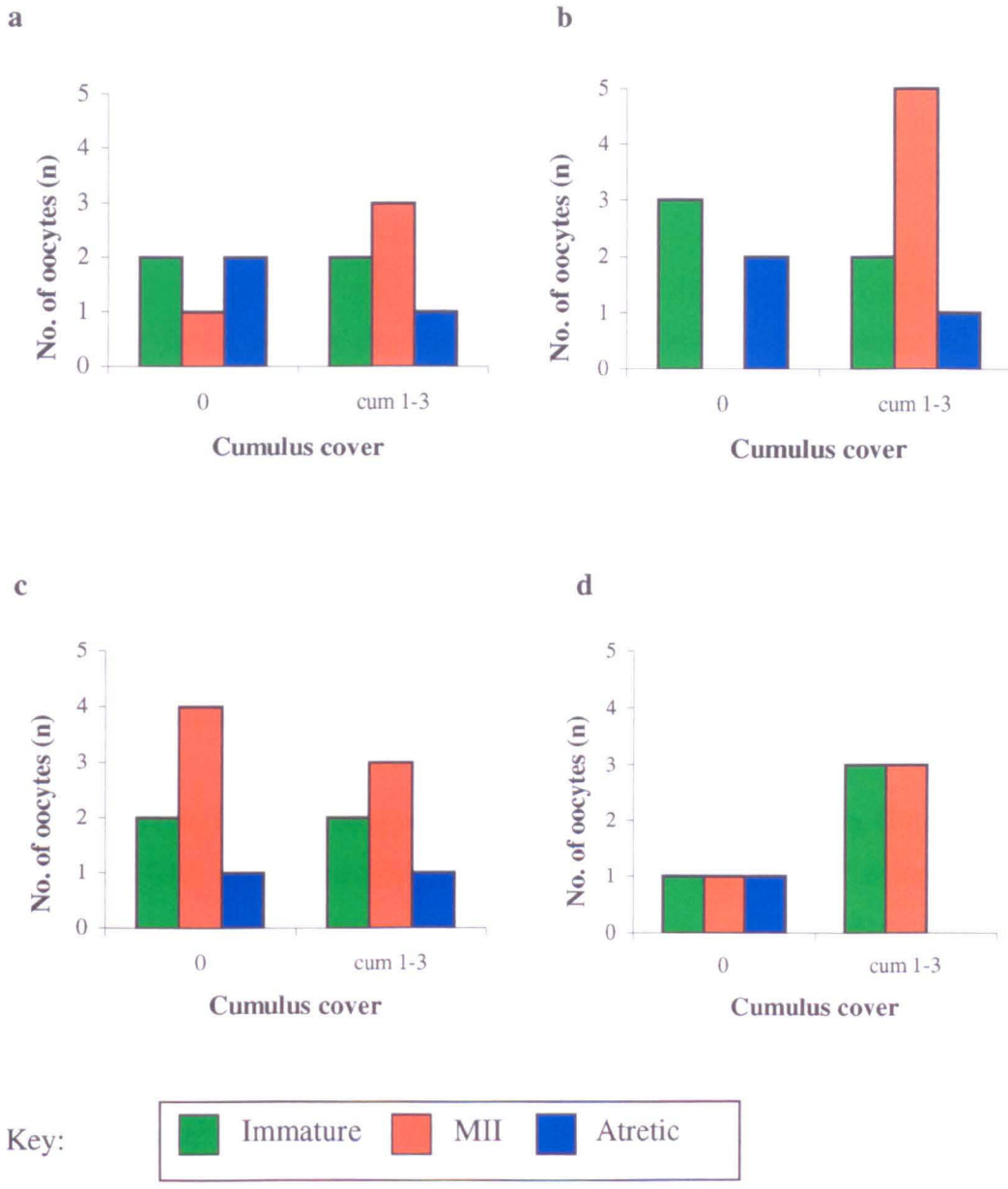


Figure 4.4 Levels of cumulus cover on oocytes on day-2 recovered from patients with polycystic ovaries (PCO) and cultured in a) vehicle, b) 0.1ng/ml EGF, c) 1.0ng/ml EGF, d) 10ng/ml EGF.

0= devoid of any cumulus/no more than 10 scattered cells; 1= partial cover; 2= complete cover; 3= substantial multilayered cover.

4.7 Discussion

The results obtained in this study generally did not indicate significant effects of EGF (at concentrations of 0.1, 1 and 10ng/ml) used alone, in the absence of gonadotrophins on in-vitro survival and maturation of human oocytes. One significant response was evident, where EGF at all concentrations combined had a positive effect upon maturation of GV oocytes ($0.025 < p < 0.05$).

A limiting factor in this experiment was the relatively low number of oocytes available (n=46), resulting in few fertilization and cleavage stage embryos for study. In view of the few significant results or trends, this part of the study was abandoned early in favour of pursuing the more fruitful work assessing the role of FF-MAS, presented in the next chapter. However, this was the first study that included the creation of embryos for research to be approved in the UK, and the establishment of methods and collection of oocytes from nine patients is a substantial number in this field, and the data adds to the pool of information available.

Das *et al.* (1991) performed a preliminary study using eight human GV oocytes and observed 100% maturation after 48 hrs culture with 5ng/ml EGF compared to 0% maturation (and signs of degeneration in one oocyte) in control. Subsequently, Gómez *et al.* (1993b) used physiological and supraphysiological concentrations of EGF similar to those used in my experiments. Three patient groups were used in their study: patients who had received no ovarian stimulation (24 oocytes obtained), patients at risk of developing OHSS and therefore having their stimulated cycles cancelled (8 oocytes) and patients undergoing HMG-hCG stimulation for IVF (93 oocytes). After 48 hrs culture of oocytes (n=24) obtained from non-stimulated ovaries, a significant difference ($p < 0.01$) was observed between the percentage of MII oocytes in control (19.2%) and in 10ng/ml EGF (54.8%), although at a physiological concentration of 2ng/ml EGF there was no significant difference. Concentrations of 2 and 10ng/ml EGF induced a borderline significant ($p = 0.07$) increase (12.5% and 12.9% respectively) on the number of oocytes reaching MII at 24 hrs when compared to control (0%). In my experiments, no oocytes reached MII after 24 hrs in culture, in agreement with Das *et al.* (1991).

The reasons for these differences might include the patient source; oocytes obtained from non-stimulated ovaries in the study by Gómez *et al.* (1993b) were obtained from 22 women undergoing gynaecological surgery for a number of benign disorders. All of these patients had spontaneous and regular menstruation, and were scheduled for surgery on days 7-10 of the menstrual cycle, which is likely to include a lower proportion of oocytes from atretic or persistent follicles than in my study. In my study oocytes were collected from mainly (75%) oligomenorrhoeic patients, therefore collection of oocytes was scheduled irrespective of the stage of the menstrual cycle. Secondly, different methods were employed to obtain the oocytes. Pieces of ovary were removed surgically, and follicles punctured, compared with my study where follicles were aspirated in vivo. Moreover, only oocyte-cumulus complexes were used in their study, whereas all viable oocytes obtained were used in my study, including those without cumulus cells.

In 1998, Goud *et al.* studied the role of cumulus cells and EGF supplementation of the culture medium on the IVM of human GV oocytes obtained after gonadotrophin stimulation for ICSI. All oocytes obtained from follicular aspirates were treated with 0.1% hyaluronidase for 1-2 min to visualize the presence of a GV. 121 oocytes from 38 patients were completely denuded of cumulus cells and cultured with or without EGF (2ng/ml). Cumulus cells were retained on 191 GV oocytes (n=54 patients), which were cultured with or without EGF (2ng/ml). More oocytes with intact cumulus reached MII by 30 hrs compared to nude oocytes, although EGF supplementation of the medium did not affect maturation of oocytes cultured with intact cumulus cells when compared to maturation in control medium lacking EGF. However, EGF did have a significant effect on promoting the maturation of nude oocytes ($p < 0.003$). They concluded that cytoplasmic maturation of human oocytes in vitro could be improved with maintenance of cumulus cells during culture and supplementation of the maturation medium with EGF. Also that the effect of EGF appears to be synergistic with gonadotrophins in the cumulus-intact oocytes.

In a study on meiotic progression in mouse follicle-enclosed oocyte culture, Smitz *et al.* (1998) observed a stimulatory effect of HCG and EGF ($\geq$ 5ng/ml) on completion of the first meiotic division, whereas EGF alone up to a dose of 50ng/ml overrode the

somatic inhibitory stimuli in fewer than half of the cultured follicles. After studying the effects of EGF on nuclear and cytoplasmic maturation of mouse oocytes grown in vivo or in vitro, De La Fuente *et al.* (1999) concluded that gonadotrophins in vivo increase the sensitivity or responsiveness of cumulus cell-enclosed oocytes to EGF, thereby promoting both nuclear and cytoplasmic maturation. However, oocyte-granulosa cell complexes grown in vitro become responsive to EGF without gonadotrophin treatment. The relationship between EGF and gonadotrophin control in respect of oocyte maturation is therefore uncertain in human oocytes and warrants further investigation.

Goud *et al.* (1998) did not observe a positive effect of EGF on the fertilization of the in vitro matured human GV oocytes without cumulus cells cultured with EGF. However, a significant positive effect ($p<0.05$) of EGF on fertilization was observed in GV oocytes obtained from the same source but cultured with cumulus cells intact.

No differences, for example in oocyte size, or size of the polar body were observed between the different culture groups. It is known that the size of a polar body can reflect the degree of peripheral movement of the spindle at MII, and disorders in spindle positioning may relate to immaturity of oocytes. Inward displacement of the spindle results in a pb of atypically large size. Oocytes with very small pbs, are associated with reduced fertilization and increased aneuploidy, since chromosomes destined for the first pb may remain entirely in the oocyte or be incompletely expelled (Veeck, 1999).

No significant effect of EGF on fertilization and cleavage was observed in my experiments, although the numbers of oocytes were low. However, cleaving embryos were achieved in all culture conditions confirming that the oocytes matured in vitro were capable of fertilization and the initial stages of embryo development, despite the absence of gonadotrophin stimulation in vitro. Early cleavage events are known to be predominantly under maternal control from inherited mRNAs (Braude *et al.*, 1988) and developmental competence is acquired sequentially as particular stages of oocyte development are reached (Eppig *et al.*, 1994)

Reduced developmental competence of human oocytes matured in vitro has been reported previously (Trounson *et al.,* 1994) and cleavage and development beyond the third cleavage division remains a problem with IVM. Cleavage arrest could result from inadequate cytoplasmic maturation, as aberrations in cytoplasmic maturation are more likely to cause embryonic demise in later stages of preimplantation development (Moor *et al.,* 1998). My data, though limited, suggest that EGF did not overcome this problem.

Although my results did not achieve significance, they tended to support the conclusion that the presence of cumulus cells was beneficial for maturation, and that EGF supplementation of the medium did not significantly affect maturation of oocytes cultured with intact cumulus cells. It is interesting to note that at a concentration of 1.0ng/ml EGF (the closest to the 2ng/ml used in Goud's study) there was an increased number of nude oocytes reaching MII when compared to nude oocytes in all other culture conditions. At this concentration only, the number of oocytes reaching maturation was higher for nude oocytes than for those with cumulus cell cover. These data confirm previous suggestion (Goud *et al.,* 1998) that EGF may be capable of acting on oocytes directly, rather than in a manner mediated by the cumulus. The relatively small EGF molecule is likely to traverse easily through the zona of human oocytes, to exert its effect directly on the oocyte (Shalgi *et al.,* 1973; Goud *et al.,* 1998).

In conclusion, my data do not indicate a significant effect of EGF on oocyte survival, maturation or development in vitro, however the size of the data set is limited. In view of the lack of significant results in this pilot study and the limited supply of human oocytes, further experiments on EGF were not undertaken. However, future directions of this research could have included, for example, increasing the numbers of oocytes in each group to assess whether significant effects upon maturation or fertilization according to EGF concentration became evident and also assessing co-supplementation of EGF with gonadotrophins which are known to stimulate maturation via cumulus cells. In addition, a comparison with oocytes that had been exposed to some in vivo stimulation might provide information on the relevance of follicular viability.

Chapter 5

Effects of FF-MAS during in vitro maturation culture

5.1 Introduction

In this part of the project, I sought to assess the effects of FF-MAS upon maturation of human oocytes in vitro, and to test the developmental competence of the matured oocytes in terms of their fertilization and embryo development. Oocytes from both patients with PCO and patients undergoing ICSI treatment were used as described in chapter 2, section 2.3. The effects of this compound were considered particularly interesting, as it is believed to be one of the endogenous factors involved in oocyte maturation in vivo, and its potential role in human oocytes had not previously been explained, despite evidence of efficacy in mice (Byskov *et al.*, 1995, 1999; Hegele-Hartung *et al.*, 1999).

Meiosis activating sterol (FF-MAS: 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol) is an intermediary occurring naturally in the biosynthetic pathway between lanosterol and cholesterol. It is present in human pre-ovulatory follicular fluid at concentrations of ~1.3μmol/l (Byskov *et al.*, 1995). It activates meiotic resumption of both cumulus-enclosed and cumulus-denuded mouse oocytes in vitro (Byskov *et al.*, 1995, 1999; Hegele-Hartung *et al.*, 1999). FF-MAS is synthesized by cumulus cells of intact oocyte-cumulus-complexes in response to FSH stimulation (Byskov 1998) and the concentrations of FF-MAS in pre-ovulatory follicular fluid samples are correlated with the ability of the associated oocyte to fertilize and cleave (Byskov *et al.*, 1998).

Grondahl *et al.* (1998) and Ruan *et al.* (1998) have demonstrated the specificity of FF-MAS as an inducer of meiosis when compared to a series of related sterol compounds. Hegele-Hartung *et al.* (1999) showed the ability of FF-MAS to stimulate GVBD in both cumulus-enclosed and denuded mouse oocytes in the presence of inhibitors such as hypoxanthine, isobutylmethylxanthine and dibutyryl cAMP (dbcAMP), which normally maintain meiotic arrest. Conflicting results from Downs *et al.* (2001) demonstrated failure of FF-MAS at a concentration of 3μg/ml to induce GVBD in denuded mouse oocytes, when meiotic arrest was maintained with dbcAMP (300μM).

However, in a parallel hypoxanthine-treated set of denuded oocytes FF-MAS at the same concentration was stimulatory.

Downs *et al.* (2001) hypothesized that the use of different culture medium may have influenced the efficacy of FF-MAS as a meiosis-inducing agent. However they concluded that the medium type did not account for their inability to demonstrate FF-MAS-stimulated maturation in dbc-AMP arrested oocytes. It was speculated that the positive action of FF-MAS on cumulus-enclosed oocytes is contingent on culture conditions, due to FSH inducing meiotic resumption in a high percentage of cumulus-enclosed oocytes, where FF-MAS had failed. Evidence lending support to this theory came from the results of glucose removal from medium, where a subsequent inhibitory effect of FF-MAS on GVBD in hypoxanthine-arrested cumulus-enclosed mouse oocytes was observed; leading Downs *et al.* (2001) to acknowledge that this change in culture medium could have altered sterol processing by the cumulus cells and thereby its influence on meiosis.

Downs *et al.* (2001) also studied the kinetics of maturation induced by FF-MAS; long-term exposure (12hr) to the sterol was required for meiotic maturation. Slow maturation kinetics (>6hr) were observed in denuded mouse oocytes cultured in 4mM hypoxanthine ± 3μg/ml FF-MAS, oocytes were assessed for GVBD at 3, 6 and 12 hr. The proportion of oocytes undergoing GVBD in the FF-MAS treated group was significantly different only at the 12hr time point. In the superovulated mouse model, GVBD is initiated between 1.5-2 hr post hCG (Eppig *et al.,* 1987). However, Downs *et al.* (2001) considered that in vitro conditions, including choice of meiotic inhibitor and absence of certain follicular constituents could alter the normal rate of maturation.

Thus, recent work by Downs *et al.* (2001), whilst confirming the stimulatory action of FF-MAS on hypoxanthine-arrested denuded mouse oocytes as discussed above; fails to support a universal meiosis-inducing function for FF-MAS. The reduced effectiveness of FF-MAS on cumulus-enclosed oocytes compared to denuded oocytes was explained by Downs *et al.* (2001) to be due in part to its sequestration by cumulus cells. The lower efficiency of FF-MAS in cumulus-enclosed oocytes when compared to denuded oocytes was also considered by Grondahl *et al.* (1998), and attributed to

either release by the cumulus cells of inhibitory factors that may negate the stimulatory action of FF-MAS or to a loss of sterol accessibility to the oocyte potentially via steroid conversions occurring in the cumulus cells (Downs *et al.*, 2001).

This chapter presents the first study of FF-MAS effects on human oocyte maturation in vitro. The results were published (Cavilla *et al.*, 2001) and the published article is presented as appendix II.

5.2 Aims

To perform a log dose assessment of the effects of FF-MAS exposure during in vitro culture of human oocytes upon their maturation, fertilization and early embryonic development. To compare the results of FF-MAS exposure in immature oocytes originating from two different patient groups: unstimulated patients with or without polycystic ovaries and patients undergoing a fully stimulated cycle of ICSI treatment.

5.3 Patients and oocytes

5.3.1 Patients

For this study, a total of 128 viable immature oocytes were collected from 19 PCO patients with unstimulated ovaries and 72 immature oocytes were donated by 28 ICSI patients. Clinical details of the patient groups are presented in Tables 3.2 and 3.3 (chapter 3).

5.3.2 Oocytes

The immature oocytes collected were randomly allocated to the three groups: 0 (control, including the vehicle EtOH, at the same concentration as the FF-MAS treatment groups), 10 and 30μg/ml FF-MAS for a period of up to 48 hrs. Oocytes were considered mature if a polar body was visible by Hoffman microscopy. All mature oocytes were subjected to ICSI with donor sperm.

5.4 Effect of MAS on survival and maturation.

Figure 5.1 shows the results of FF-MAS exposure of oocytes obtained from PCO patients, in terms of oocyte survival and maturation, according to the concentration of FF-MAS present in the culture medium during the period of maturation culture. FF-MAS at a concentration of 30μg/ml significantly increased oocyte survival ($P< 0.025$, $p \times q$ χ^2 test, Campbell, 1989) resulting in 88% survival in 30μg/ml compared with 63% and 60% for 10μg/ml and control respectively. The increased survival of oocytes in 30μg/ml FF-MAS was not associated with an alteration in the proportion of surviving oocytes, which matured which was 42%, 37% and 48% in 30μg/ml, 10μg/ml FF-MAS and control respectively.

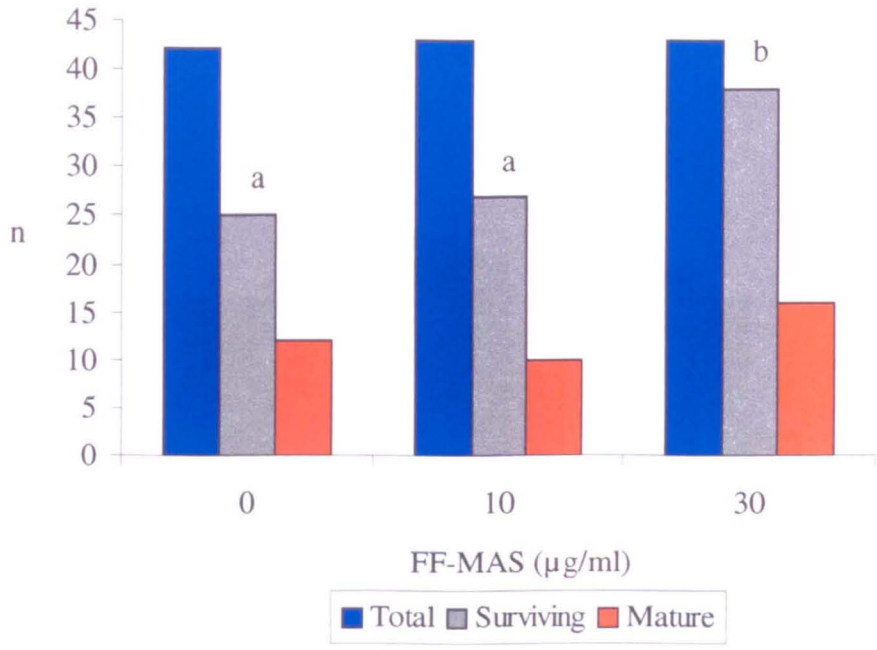


Figure 5.1 In-vitro maturation of immature oocytes (n=128) collected from patients (n=19) with polycystic ovaries and cultured with or without meiosis activating sterol (FF-MAS).
Bars with different letters are significantly different ($P < 0.025$).

Figure 5.2 shows the results of FF-MAS exposure of immature oocytes from ICSI patients. Oocyte survival was >90% in all groups, regardless of FF-MAS concentration. However, in this patient group, the presence of MAS at 10 or 30μg/ml significantly increased the proportion of oocytes maturing in vitro ($P< 0.05$, $p \times q$ χ^2 test, Campbell, 1989).

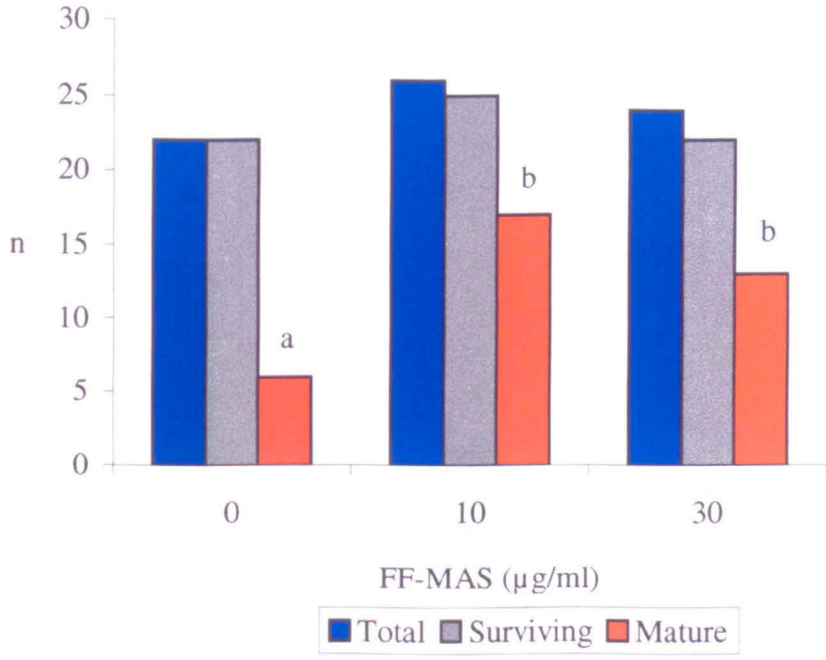


Figure 5.2 In-vitro maturation of immature oocytes (n=72) donated by patients undergoing intracytoplasmic sperm injection (ICSI) (n=28) and cultured with or without meiosis activating sterol (FF-MAS).
Bars with different letters are significantly different ($P < 0.05$).

Figure 5.3 shows the stage of maturity of the oocytes collected from the two patient groups, patients with PCO and patients undergoing ICSI treatment. Significantly more immature oocytes per patient were obtained from PCO patients in comparison with ICSI patients ($p< 0.01$) as would be expected, but surprisingly there was no significant difference in the proportion of immature oocytes that had undergone GVBD at the time of oocyte collection between the PCO and ICSI patient groups. Oocytes in which the nuclear status was obscured by the tight covering of cumulus cells were assumed to be at the GV stage, and identified separately on the graph.

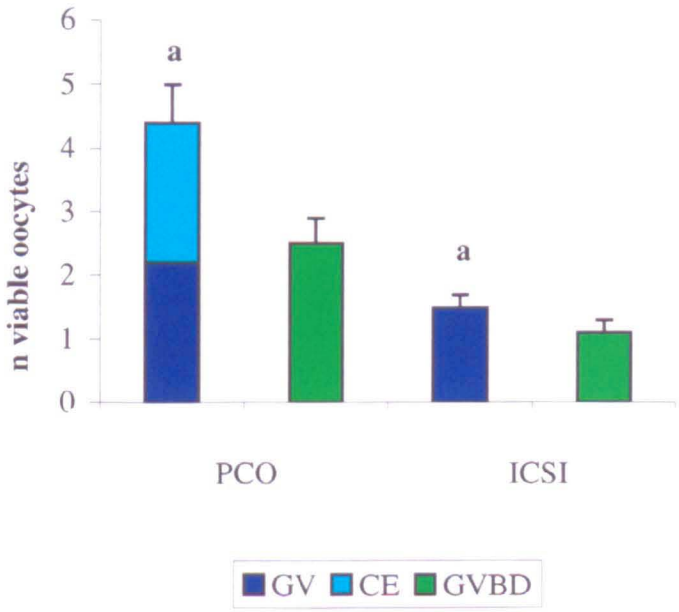
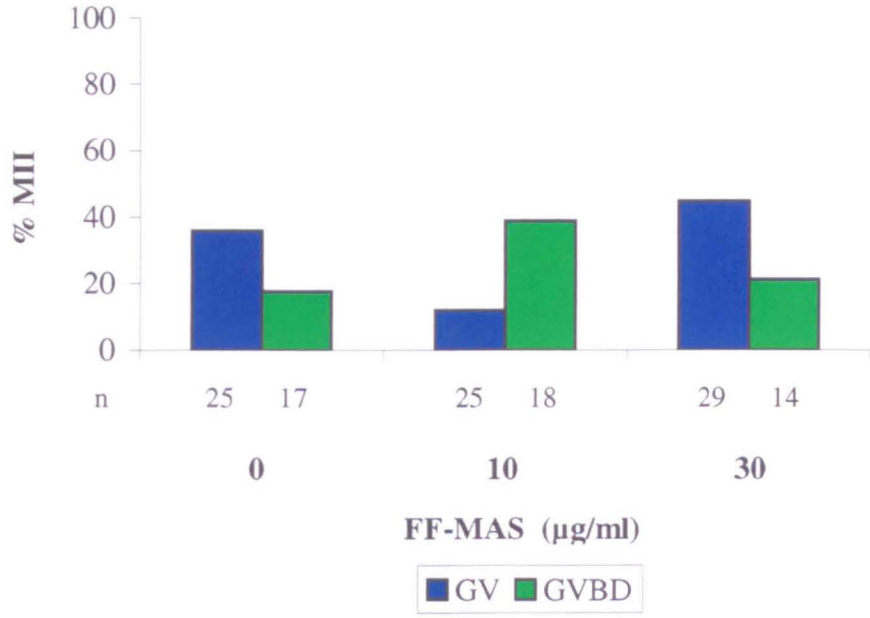


Figure 5.3 Maturity at collection of oocytes recovered from polycystic ovaries (PCO) and intracytoplasmic sperm injection (ICSI) patients.
GV=germinal vesicle; CE=cumulus enclosed (GV assumed, but not visible); GVBD=germinal vesicle breakdown.
Results are expressed as mean ± SEM, [a] significantly different ($P< 0.01$).

Figures 5.4 (a) and (b) show the extent of maturation in vitro of oocytes collected at the GV and GVBD stages for both patient groups. In PCO patients Figure 5.4 (**a**), the proportion of oocytes in both categories reaching MII was highly variable. Cultured immature oocytes (either GV or GVBD at collection) from ICSI patients had a low maturation rate but the maturation rate of GVBD oocytes was significantly improved by addition of FF-MAS ($p< 0.025$, χ^2 test), as shown in Figure 5.4 (b). For the oocytes that failed to mature in the ICSI group, approximately equal numbers arrested at or after the GV stage.

Figure 5.5 shows the incidence of atresia occurring in vitro after collection of apparently viable oocytes. Especially high levels of atresia were noted in GVBD oocytes from PCO patients. Overall the PCO oocytes appeared more susceptible than ICSI oocytes. In the ICSI group, atresia was confined to oocytes collected at the GV stage.

a



b

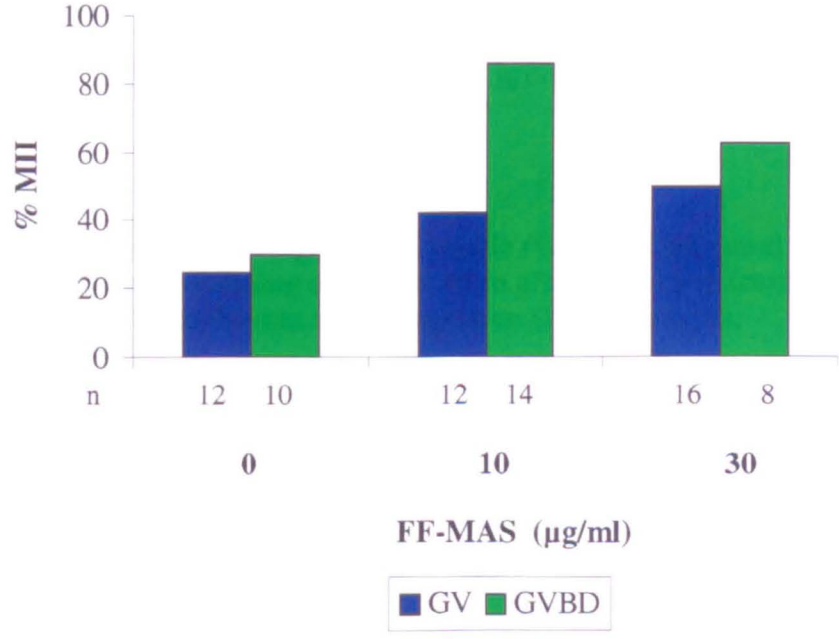


Figure 5.4 Maturation of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) oocytes from (a) patients with polycystic ovaries (PCO) and (b) patients undergoing intracytoplasmic sperm injection (ICSI) in the presence or absence of meiosis activating sterol (FF-MAS).

n= number of oocytes in each group

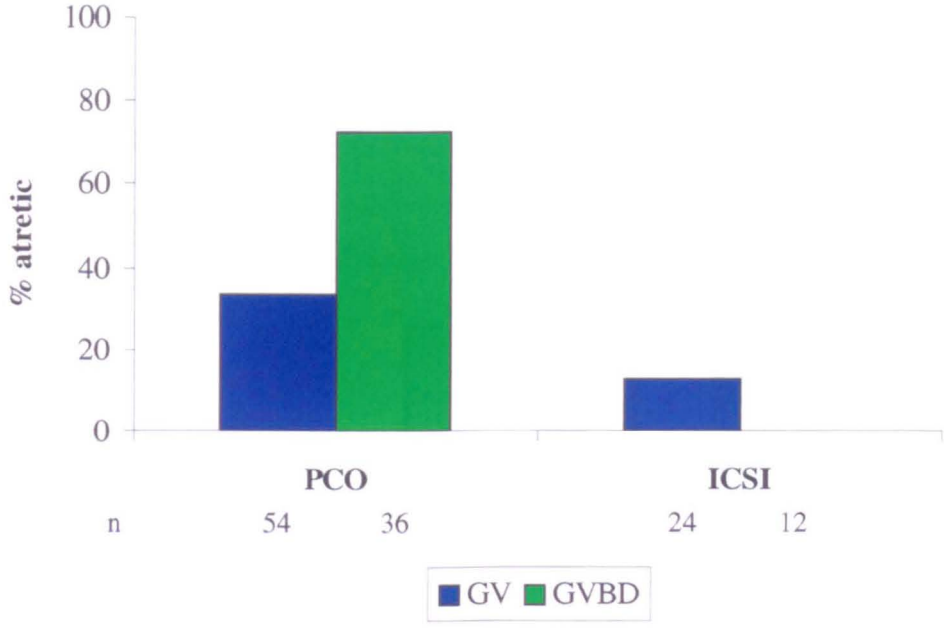


Figure 5.5 Proportions of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) oocytes becoming atretic in vitro after collection from polycystic ovaries (PCO) or intracytoplasmic sperm injection (ICSI) patients.

n= number of oocytes in each group

The time course of oocyte maturation differed between the two patient groups. Of those oocytes maturing to MII in vitro, 50% in the ICSI group had reached MII by 23-24 hr compared with <5% for PCO patients as shown in Figure 5.6. The majority (89.2%) of PCO oocytes that matured to MII did so on the second day of IVM culture.

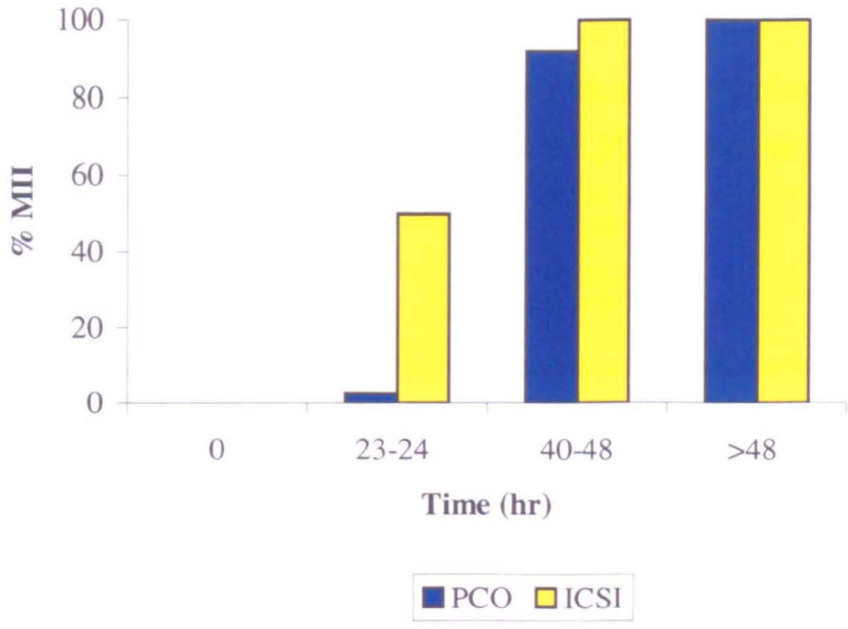


Figure 5.6 Cumulative time course of maturation in vitro for maturing oocytes from polycystic ovaries (PCO) and intracytoplasmic sperm injection (ICSI) patients.

5.5 Effects of FF-MAS on fertilization and embryo development

The proportions of in-vitro matured oocytes fertilized by ICSI and subsequently cleaving in either PCO or ICSI patient groups are presented in Tables 5.1 and 5.2. The fertilization rate was defined as the number of 2PN oocytes as a proportion of the oocytes surviving ICSI. Cleavage rate was defined as the number of cleaving embryos as a proportion of the 2PN oocytes. The fertilization rates achieved were broadly similar in the two patient groups.

In both patient groups, oocytes cultured with FF-MAS showed a tendency towards improved subsequent development, however these data were not tested statistically in view of the low numbers of oocytes reaching this stage. No blastocysts were formed and the most advanced embryos arrested at the 3rd cleavage division. The stages of arrest are indicated in parentheses in table 5.2.

Table 5.1 Fertilization rates of in-vitro matured oocytes arising from patients with polycystic ovaries (PCO) or those being treated with intracytoplasmic sperm injection (ICSI).

	FF-Meiosis activating sterol (μg/ml)		
	0	10	30
PCO	**4/10** (40%)	**2/9** (22%)	**7/12** (58%)
ICSI	**2/5** (40%)	**6/11** (55%)	**6/10** (60%)

Table 5.2 Cleavage rates of in-vitro matured oocytes arising from patients with polycystic ovaries (PCO) or those being treated with intracytoplasmic sperm injection (ICSI).

	FF-Meiosis activating sterol (μg/ml)		
	0	10	30
PCO	**2/4** (3c, 4c)	**2/2** (3c, 6c)	**5/7** (2x3c, 2x4c, 5c)
ICSI	**0/2**	**3/6** (2c, 3c, 5c)	**5/6** (2c, 3c, 4c, 5c, 6c)

Table 5.3 shows the stage and grade of embryos arising from the fertilization of in-vitro matured oocytes retrieved from patients with polycystic ovaries (PCO) and from patients undergoing ICSI treatment. Oocytes were graded according to the grading system devised by Veeck (1990) (Chapter 2, section 2.5.2) with grade 1 representing ideal morphology. Embryos resulting from the fertilization of in vitro matured oocytes retrieved from patients with PCO and those undergoing ICSI were on average grade 3. Oocytes matured in 30μg/ml FF-MAS in both patient groups appeared to result in embryos of a better quality (ranging from grade 2+ - 3), although this was not a significant finding, possibly in view of the limited numbers of embryos for analysis.

Table 5.3 Stage and grade of embryos arising from fertilization of in-vitro matured oocytes retrieved from patients with polycystic ovaries (PCO) or those being treated with intracytoplasmic sperm injection (ICSI).

	FF-Meiosis activating sterol (μg/ml)		
	0	10	30
PCO	3c **(4)** 4c **(3)**	3c **(2)** 6c **(4)**	3c **(3)** 3c **(3+)** 4c **(3+)** 4c **(3)** 5c **(3)**
ICSI		2c **(4)** 3c **(3)** 5c **(4)**	2c **(3)** 3c **(3)** 4c **(2)** 5c **(3)** 6c **(2+)**

Grade of embryo indicated in parentheses

Of the nine cleavage stage embryos arising from fertilization of in vitro matured oocytes obtained from patients with PCO, one was damaged during the staining procedure. Of the remaining eight embryos, six had one nucleus per cell as would be expected for normal development. One 3-cell embryo had only two nuclei, and one 5-

cell embryo had only four nuclei, indicating the occurrence of cell division without nuclear division.

Of the eight cleavage stage embryos arising from fertilization of in vitro matured oocytes obtained from patients undergoing ICSI treatment, six had one nucleus per cell. One 5-cell embryo had only 3 nucleate cells and the 6-cell embryo had five nuclei, with two of the nuclei in one cell, therefore two cells were anucleate.

Once oocytes had matured, there were some visible differences in polar body (pb) size and shape within each patient group (table 5.4 and table 5.5).

Two cases of abnormal fertilization were observed in oocytes obtained from patients with PCO cultured in control and 10μg/ml FF-MAS: four pronuclei were seen after ICSI in an oocyte matured in control conditions and three pronuclei were seen after ICSI in an oocyte matured in 10μg/ml FF-MAS. Both of these oocytes had only one pb (indicating digyny), and small pronuclei. An inverse correlation between pronuclear number and size is documented (Veeck, 1999) and pronuclear fragmentation may also have occurred. The 4PN zygote had a first pb of normal appearance, but the 3PN zygote had a flattened, fragmented pb. The majority of oocytes with normal polar bodies fertilized normally or failed to fertilize, however, no oocytes with either small or large first pbs fertilized.

A total of nine oocytes had fragmented first pbs, these arose in all culture groups, although over half (56%) had matured in 10μg/ml FF-MAS; interestingly this corresponded with the lower fertilization score (22%) out of the three culture conditions for the PCO patient group, however it is unclear whether this association is causative. In the PCO patient group, 16.7% of oocytes that matured without FF-MAS (control) underwent atresia after ICSI compared to 15.4% in the combined FF-MAS culture groups. This was not significantly different.

Table 5.4 Polar body types observed in mature oocytes obtained from patients with PCO, cultured with or without FF-MAS

Polar body	Outcome of ICSI			
	2 pn	>2pn	Unfertilized	Atretic
Normal	10	1	10	2
Small			2	1
Large				2
Fragmented	3	1	5	
Flattened				1

Among oocytes from ICSI patients, one case of abnormal fertilization was observed in an oocyte matured in 10μg/ml FF-MAS, where four small pronuclei and two pbs were observed. This zygote did not develop any further. Around half of the oocytes with normal pbs (arising from maturation in all culture groups) failed to fertilize. Five of the nine unfertilized oocytes had no membrane resistance when injecting sperm. A total of seven oocytes had fragmented first pbs and these arose from all culture groups; however only one oocyte was matured in control conditions and this had a slightly fragmented pb. Four of these oocytes fertilized normally and three of the resulting embryos cleaved. Two oocytes matured in FF-MAS had flattened pbs, but fertilized after ICSI.

In this series of oocytes from ICSI patients, 16.6% that had matured without FF-MAS (control) underwent atresia after ICSI compared to 20% in the combined FF-MAS culture groups. This was not significantly different.

Table 5.5 Polar body types observed in mature oocytes donated by patients undergoing ICSI treatment, cultured with or without FF-MAS				
Polar body	Outcome of ICSI			
	2 pn	>2pn	Unfertilized	Atretic
Normal	8	1	9	5
Small				
Large				
Fragmented	4		1	2
Flattened	2		1	

In both patient groups, the incidence of atresia after ICSI was apparently higher than the normal ~10% atresia level expected with fresh oocytes in ICSI treatment. This is probably due to fragility of the oocytes, possibly cause by prolonged IVM culture, however three injections resulting in atresia of the oocyte were definitely due to poor injections.

In the ICSI patients, 33% of oocytes that matured early (23-24 hr) and were therefore injected with sperm after 24 hr (rather than being aged a further day) fertilized after ICSI compared with 44% of oocytes maturing after 40-48 hr culture. This was not a significant difference. Of the oocytes that matured early, 61% were cultured in 10μg/ml FF-MAS; the highest proportion (44%) maturing in the 40-48 hr culture time was cultured in 30μg/ml MAS. When comparing the early (23-24 hr) and the 40-48 hr culture periods, there were no significant differences between the numbers failing to fertilize and becoming atretic.

5.6 Effect of cumulus cells on oocyte development

Table 5.6 shows the extent of oocyte maturation according to the amount of cumulus cell cover at oocyte recovery. In all culture conditions, the maturation rate was approximately doubled in oocytes with cumulus cover compared to nude oocytes ($p < 0.025$) and atresia appeared less likely in those with cumulus cover (approximately halved) than in those without, in all culture conditions. Statistical

analysis was not performed on the latter comparison since the presence of a zero in the data set did not meet the conditions for contingency tests.

Table 5.6 Effects of cumulus presence at collection (day-2) of oocytes recovered from patients with polycystic ovaries (PCO) and cultured in 30μg/ml FF-MAS, 10μg/ml FF- MAS or control.

Culture	Cumulus grade	Mature	Immature	Atretic
Control	0	**19%** (4/21)	**19%** (4/21)	**62%** (13/21)
	1-3	**38%** (8/21)	**43%** (9/21)	**19%** (4/21)
10μg/ml FF-MAS	0	**17%** (4/23)	**35%** (8/23)	**48%** (11/23)
	1-3	**30%** (6/20)	**45%** (9/20)	**25%** (5/20)
30μg/ml FF-MAS	0	**24%** (5/21)	**52%** (11/21)	**24%** (5/21)
	1-3	**50%** (11/22)	**50%** (11/22)	0

0 = devoid of any cumulus/ no more than 10 scattered cells; 1 = partial cover;
2 = complete cover; 3 = substantial multilayered cover.

5.7 Discussion

My results demonstrate significant effects of FF-MAS on in vitro survival and maturation of human oocytes. Immature oocytes collected from two different patient groups, unstimulated PCO patients and stimulated ICSI patients, have been shown to differ in terms of oocyte function in vitro, including the proportions maturing and the speed of maturation in vitro. These differences may reflect patient-related differences in the origin of the oocytes, including the stage of follicular development at oocyte retrieval, the endocrine status or atresia of the follicles and the presence or absence of supporting cumulus cells. These results will be important for the future application of IVM as a potential treatment for certain forms of infertility. While IVM has been used with varying degrees of success in Australia, USA and Scandinavia, it has only recently been considered by the HFEA as a potential treatment and the first UK treatment licenses are yet to be approved. This study was specifically required by the HFEA to focus upon data collection on research embryos as a pilot study of this nature was essential before any treatment licence application would be considered.

FF-MAS significantly ($P< 0.025$) improved the survival of immature oocytes collected from unstimulated women with PCO. This is in contrast with the results obtained culturing immature oocytes from stimulated ICSI patients, in whom FF-MAS did not affect oocyte survival (which was >90% in this patient group), but maturation in vitro was significantly increased ($P< 0.05$). The variations in embryo development in both patient groups with FF-MAS supplementation of IVM media are also interesting, particularly in view of the compromised developmental competence which is widely believed to be associated with immature oocytes collected from PCO patients (Almahbobi *et al.*, 1996). Although statistical analysis of developmental outcome in vitro was precluded by the limited numbers of oocytes reaching the later stages in this study. The limited availability of human oocytes for research is widely known, and hence this study describes one of the largest data sets available, conducted purely for research.

FF-MAS is present in human pre-ovulatory follicular fluid at a concentration of ~1.3μM. In this study supraphysiological concentrations of FF-MAS were employed in vitro, 3μg/ml (7.3μM) and 30μg/ml (73.2μM). These higher concentrations of FF-

MAS were used for IVM because it is not known how much steroid actually reaches the oocyte in vitro or the route by which it travels (Downs *et al.,* 2001). The concentration of FF-MAS in the IVM medium may be much reduced, for example by steroid adhesion to the plastic culture vessel or by sequestration into the cumulus cells.

FF-MAS is also produced in cumulus cells, which are steroidogenically competent, in common with other somatic cell components of the follicle (Leonardsen *et al.,* 2000). Its effects are likely to be mediated via mechanisms employed by other follicular steroids, perhaps in a manner similar to progesterone stimulation of meiosis in *Xenopus* oocytes (where progesterone acts through a membrane-bound receptor) (Byskov *et al.,* 1998). The FF-MAS receptor and the signalling pathways for FF-MAS during resumption of oocyte meiosis have not yet been clarified. However, Janowski *et al.* (1996) observed FF-MAS to be a ligand for the orphan nuclear receptor LXR-α, and Grondahl *et al.* (2000) suggested that a putative nuclear MAS receptor could have similarities at least in the ligand-binding domain to LXR-α.

The steroid environment of follicles in PCO is disturbed (e.g. Almahbobi and Trounson, 1996; Pierro *et al.,* 1997), and so the influence of FF-MAS in this environment might differ from other circumstances. Such as, for example, after ovarian stimulation with gonadotrophins, which would support several follicles concurrently in a non-atretic state.

Oocytes with cumulus cells attached, retrieved from patients with PCO exhibited a significantly higher maturation rate than oocytes with no cumulus cells in all culture conditions, (χ^2 test, $p< 0.025$). An increased likelihood of maturation in the presence of cumulus cover confirms findings by Wynn *et al,* (1988). However, others (Barnes *et al,* 1996; Russell, 1998) have found similar rates of maturation and fertilization in compact cumulus enclosed oocytes and nude oocytes, although Russell (1998) reported a higher cleavage rate in oocytes with compact cumulus cover. Downs *et al.* (2001) suggested that FF-MAS was not as effective in cumulus-enclosed mouse oocytes as it was in denuded oocytes, possibly due to cumulus cells suppressing the uptake of sterol intermediates by the oocyte, or converting FF-MAS to another

steroid, however, FF-MAS appeared more effective on the cumulus-enclosed oocytes in my study. The differences between mouse and human oocyte maturation may be one factor contributing to this observation. Immature oocytes retrieved from unstimulated PCO patients may or may not have cumulus cells covering them. The presence or absence of cumulus cells provides some indication of the follicle from which the oocyte originated, denuded oocytes being more likely to have arisen from follicles becoming atretic. The known roles of cumulus cells include supporting oocyte growth and maintaining meiotic arrest throughout all immature stages of oocyte development. They are steroidogenically active and also produce hyaluronic acid during cumulus expansion to facilitate the approach of spermatozoa to the zona pellucida at fertilization. The somatic cells may also offer some non-specific support e.g. potentially buffering against sub-optimal culture media or osmotic shock. Oocytes surrounded by cumulus cells may therefore be at an advantage during IVM culture.

Most enclosed oocytes had compact corona cells and contained a GV, and many of those without corona cells had progressed to GVBD, probably as a result of incipient atresia in the follicle (Gougeon and Testart, 1986; Anderson *et al.*, 1997). Surprisingly, similar proportions of oocytes from PCO and ICSI patients had a GV or had undergone GVBD; however, these oocytes differed in their tendency towards atresia and their ability to mature to MII suggesting that those undergoing GVBD in the two groups were not similar populations. In addition, the removal of cumulus cells from immature oocytes collected from ICSI cycles might have affected some aspects of their development.

Several studies have shown that aspirated oocytes may be in various stages of maturation (Veeck *et al.*, 1983; De Vos *et al.*, 1999). Oocytes that remain immature having failed to respond to ovarian stimulation in vivo (such as those derived from patients undergoing ICSI treatment in this study) may be of inherently reduced quality, possibly derived from small follicles with reduced gonadotrophin sensitivity or having never reached meiotic competence. Alternatively, they may have arisen from otherwise normal follicles, which did not receive the same hormonal stimulus as others, perhaps due to their relative position in the ovary or limited blood supply (Van

Blerkom *et al.*, 1997). Moreover, the artificial conditions of gonadotrophin stimulation may have affected some aspects of their metabolism or development (Johnson *et al.*, 1991). Nevertheless, it has been reported that these oocytes are capable of maturing in vitro, fertilizing and developing normally (Cha and Chian, 1998). My results demonstrate their responsiveness to stimulation with FF-MAS, despite the absence of cumulus cells and their prior exposure to ovarian stimulation.

The importance of the endocrine environment in ensuring normal cytoplasmic maturation and subsequent fertilization is well known from work in animals (Moor and Trounson, 1977; Anderiesz and Trounson, 1995). The differing results obtained in this study in patients with differing endocrine profiles underlies the potential for IVM success rates to be affected by the oocytes' prior exposure to physiological or pathological hormonal environments. The lower maturation rate observed in the oocytes originating from unstimulated patients with PCO was probably due to compromised follicle development as a result of the abnormal endocrine environment, which could also account for the higher rate of atresia observed in the GVBD oocytes, compared with the ICSI patient group. In PCO patients, GVBD oocytes were probably retrieved from partially atretic follicles where granulosa cells have dissociated from the oocyte and factors controlling meiotic arrest have been lost. Oocytes in atretic follicles may often be found to have progressed further than meiotic prophase I (Anderson *et al.*, 1997).

In vitro, 28-30 hr is required for the maturation of unstimulated human GV oocytes to MI and 36-37 hr to MII (Edwards, 1965a, b). After HCG injection to simulate an LH surge, the results are similar, the majority of the oocytes having extruded the first polar body by 36 hr after HCG injection (Janssenswillen *et al.*, 1995). One group, (Jamieson *et al.*, 1991) reported significantly lower IVF and cleavage rates in oocytes retrieved <36 hr after the luteinizing stimulus, demonstrating the importance of appropriate timing of insemination. Previous studies have shown that immature oocytes obtained from stimulated cycles are more likely to undergo maturation than those from unstimulated cycles and that the time required for their maturation from GV to MII is reduced (Gomez *et al.*, 1993a; Cha and Chian, 1998). Others have shown the relative effects of follicular versus luteal phase retrieval of oocytes (Cha

and Chian, 1991; Whitacre *et al.*, 1998), underlining the importance of the endocrine environment. In my study, PCO patients were not given any form of hormonal manipulation prior to oocyte retrieval; however it is likely that induction of a withdrawal bleed with or without late follicular administration of HCG (Buckett *et al.*, 1999; Chian *et al.*, 1999) may improve the maturation rates achieved in this study. These methods still have the benefit of avoiding the major element of the gonadotrophic drugs normally administered for ovarian stimulation.

Throughout this series, the use of oil as a medium overlay was avoided, as oil would have extracted the steroidal FF-MAS. However, the possibility of FF-MAS adhering to the plastic culture vessel or being affected by albumin or other constituents in the culture medium cannot be excluded. Similarly, the method of dissolving steroids in EtOH is widely employed, but recent evidence has demonstrated that even concentrations <1% EtOH can adversely affect bovine IVM and subsequent embryo development (Avery and Greve, 2000). Bovine oocytes contain more lipidic yolk-like granules than human oocytes; however it is possible that some of the limited development observed in my study may relate to the use of EtOH in the cultures. This would not, however affect the validity of the results observed, since EtOH was included at a similar concentration in all cultures. Downs *et al.* (2001) confirmed results found in previous studies (Grondahl *et al.*, 1998; Ruan *et al.*, 1998; Hegele-Hartung *et al.*, 1999) that concentrations of EtOH present in the active FF-MAS preparations (0.05-0.5%) had no significant impact on meiotic maturation, however higher concentrations stimulated GVBD in hypoxanthine-arrested denuded mouse oocytes. Downs *et al.* (2001) concluded that the meiosis-inducing action of FF-MAS is a specific one and is not likely to be due to an EtOH effect, although they recommended that caution be exercised when using EtOH as a vehicle in oocyte maturation experiments, particularly those using denuded oocytes in view of the studies on bovine oocytes (Avery *et al.*, 1999; Avery and Greve, 2000). An alternative method for solubilization of MAS, using several 1-min sonication pulses (Byskov *et al.*, 1995; Leonardsen *et al.*, 2000) is referred to by Downs *et al.* (2001), however they conclude that the effects, if any, of the sonication procedure on the other constituents in the maturation medium remain to be determined.

IVM, when applied as a treatment for infertility can result in pregnancy (Cha *et al.*, 1991; Trounson *et al.*, 1994; Barnes *et al.*, 1995; Russell *et al.*, 1998; Jaroudi *et al.*, 1999;) but success rates remain lower than those of in-vivo stimulated cycles, indicating that optimization of IVM remains a challenge (Goud, *et al.*, 1998). The primary problem in oocytes matured in vitro is reduced developmental competence, particularly cleavage and development beyond the 4- cell stage (Trounson *et al.*, 1994). This was apparent in my study, the most advanced embryo observed reached 6 cells with most embryos arresting around the second cleavage division (3-4 cells). However, it appears that if the initiation of maturation is triggered in vivo, then developmental potential increases (Chian *et al.*, 1999a,b) although the value of priming with low-dose FSH remains unclear (Wynn *et al.*, 1998; Mikkelson *et al.*, 1999).

In my study, the effects of FF-MAS alone have been studied, although addition of FSH to the medium might augment FF-MAS production in the cumulus (Byskov *et al,* 1997) and the inclusion of FSH, HCG and/or growth factors in vitro has already been demonstrated to stimulate IVM by others (Durinzi *et al.*, 1997). It was decided to omit these other factors so that the effects of FF-MAS alone could be established in the absence of confounding influences. This was deemed to be important, even though a higher overall maturation rate might be gained by a combination of supplements in the medium.

Grøndahl *et al.* (2000) presented the results of IVM of 81 human oocytes allocated into seven groups, with or without synthetic FF-MAS, for histological analysis. These data showed a significant ($p< 0.05$) increase in the proportion of immature oocytes completing maturation after 30 hr in vitro in the presence of 20μmol/l FF-MAS; however, at 22 and 40 hr, the difference was not significant. Grøndahl's study differed from mine in several respects. His patient group had polycystic ovaries, but received oral contraception for 2 months, to which was added a gonadotrophin-releasing hormone (GnRH) agonist for pituitary down-regulation, followed by recombinant FSH for 3 days. Follicles of 8-12mm were aspirated on days 7-9 and all oocytes were cumulus-enclosed throughout culture. These patients therefore underwent stimulation cycles. Also, half their aspirated oocytes were used for infertility treatment, although

HCG was not administered. The medium used by Grøndahl *et al.* for the research oocytes was similar to that which I used, being M199 supplemented with 0.29 mmol/l pyruvate, antibiotics and 0.8% HSA, with or without FF-MAS which was prepared in EtOH. None of their research oocytes was inseminated. It is not yet known whether embryos resulting from IVM in the presence of FF-MAS have the potential to develop further or implant.

It has been reported that extracytoplasmic anomalies such as first pb morphology at time of ICSI are indicative of subsequent fertilization and embryo quality (Xia 1997; Ebner *et al.*, 2000). In my study, first pb morphology was variable in the patients with PCO and did not relate to the ability of the oocyte to fertilize or embryo quality. Oocytes with normal (intact, well-shaped ovoid with smooth surface) or fragmented first pbs resulted in average grade 3 embryos (according to Veeck embryo scoring method, 1990), and there was no relationship between first pb morphology and fertilization rates. In the ICSI patients, oocytes with normal first pbs resulted in embryos with an average of grade 2, however oocytes with abnormal fragmented first pbs resulted in embryos with an average of grade 4, lending more support to the data collected by Ebner *et al.* (2000).

Mikkelson *et al.* (2001) investigated the morphology of in vitro-matured oocytes and impact on fertilization potential and embryo quality, and found an association between first pb and embryo quality. A significantly reduced quality of embryos was observed when embryos developed from oocytes with one or more anomalies (such as fragmented first pb and/or a large perivitelline space) were compared with embryos developed from normal oocytes ($p=0.001$). Previously in 1995, Eichenlaub-Ritter *et al.* (1995) suggested that degeneration of the first pb may be due to asynchrony of nuclear and cytoplasmic maturation. According to Veeck (1999), oocytes with fragmented and/or flattened first polar bodies (pbs) may demonstrate a reduced potential for development and are often associated with a higher incidence of triploidy. Variation in the size of the first pb (overly large or small) may represent an abnormal condition, inward displacement of the spindle results in a pb of atypically large size. In this study, among oocytes retrieved from patients with PCO none with either small or large first pbs fertilized after ICSI. However, three out of nine (33%)

oocytes with fragmented first pbs fertilized, which is similar to the fertilization rate of 43% of oocytes with normal first pbs. Among oocytes retrieved from patients undergoing ICSI treatment, four out of seven (57%) oocytes with fragmented first pbs fertilized compared to a fertilization rate of 35% in oocytes with normal first pbs. Fragmented first pbs did not appear to affect the fertilization rate of oocytes in this study.

Studies on IVM (without FF-MAS) and resulting embryos have shown a higher incidence of nuclear abnormalities when compared to those derived from in-vivo matured oocytes. DeScisciolo *et al.* (2000) compared nuclear morphology of embryos using FISH for chromosomes X, Y and 18 to compare rates of aneuploidy, mosaicism and nuclear abnormalities. Although no difference in the rate of aneuploidy or in incidence of mosaicism was demonstrated, significantly fewer embryos (3% vs. 23%) arising from IVM oocytes were classified as normal based on nuclear morphology.

In my study, oocyte maturation with development as far as the second cleavage division has been demonstrated. Only one of the eight cleavage stage embryos (6-cell) arising from the fertilization of in vitro matured oocytes obtained from patients undergoing ICSI treatment, exhibited a multinucleated blastomere. Multinucleation occurs both in vivo and in vitro and it may serve as a mechanism for ridding the preembryo of defective blastomeres. These cells most likely represent random failures of cytokinesis (Veeck, 1999). The converse, cytokinesis without karyokinesis, resulting in cells lacking nuclei was also observed in two cleavage stage embryos arising from the fertilization of in vitro matured oocytes obtained from patients with PCO and in one cleavage stage embryo arising from the fertilization of in vitro matured oocytes obtained from patients undergoing ICSI treatment. The size of the cells appeared normal for the developmental stage and so these anucleate cells were not considered on visual inspection to be fragments. The effects of FF-MAS on embryo development could not be analyzed statistically in view of the low numbers of embryos generated.

Evidence is accumulating that FF-MAS is an important endogenous factor involved in promoting oocyte maturation. The data presented here show positive effects of FF-

MAS upon survival and maturation of human immature oocytes collected from unstimulated PCO patients and stimulated ICSI patients respectively. Oocytes derived from stimulated ICSI patients and unstimulated PCO patients constitute different populations, probably due to their differing endocrine and intrafollicular environments. FF-MAS exerted positive effects upon different aspects of oocyte function in both groups. In view of its role as an intrafollicular steroid, and its ability to promote maturation, its potential for further development as an in-vitro meiotic stimulant should be evaluated further.

If MAS is the natural paracrine hormone that triggers the resumption of oocyte meiosis or spermatogenesis, treating women and men with either agonists or antagonists to MAS could potentially control these processes, thus stimulating or blocking MAS action (Byskov *et al.*, 1998). MAS could potentially be used to promote fertility in certain conditions, i.e. men with a low sperm production, could be treated with MAS/MAS agonists, which might induce a higher number of spermatogonia to enter meiosis. Furthermore, if an antifertility drug could be developed based on controlling the MAS receptor response, it would be unlikely to affect the normal hormonal balance or interfere with normal steroid synthesis, as it would not be based on sex steroids (Byskov *et al.*, 1998). While there is great potential for MAS action in fertility, the complexity of the system ensuring that reproduction continues involves many parallel and redundant pathways, so the efficacy of such an approach would require through evaluation.

In view of the limited developmental competence of embryos observed, and the variable potential for atresia in the oocytes from PCO patients, I planned to examine the normality of the maturing oocytes by direct means in the next set of experiments.

Chapter 6

Assessments of normality of maturation in vitro: metaphase spindles and chromosome assessments

6.1 Introduction

The process of maturation encompasses a complex series of molecular and structural events, culminating in the arrest of the oocyte with chromosomes on the metaphase II spindle in anticipation of sperm penetration and activation for fertilization (Trounson, 2001).

It is well documented that oocytes can spontaneously progress through the nuclear changes characteristic of oocyte maturation when liberated from the antral follicle and cultured in vitro (Pincus and Enzmann, 1935; Edwards, 1965a,b). However, this process may be less physiological than that occurring in follicles receiving appropriate signals in vivo. Prior to meiotic maturation the GV or oocyte nucleus is large, pale, spherical and contains a single, exocentric nucleolus. According to Mattson and Albertini (1990), who studied chromatin and microtubule dynamics during meiotic prophase in the mouse, there are four patterns of chromatin organization in the GV oocyte. This reorganization of GV chromatin occurs during the preantral to antral follicle transition, and the sequential alterations (stage I-IV) in chromatin reorganization occur sequentially in vitro prior to GVBD and the spontaneous resumption of maturation. Similar stages of chromatin development are observed in human oocytes, although the timing of their occurrence in relation to follicle development has not been documented. Chromatin foci appear at the nucleolar periphery (stages I-III), stage IV is when the foci coalesce to envelope the nucleolus.

- Stage I: 6-10 chromatin foci, homogenous staining throughout nucleoplasm
- Stage II: 2-3 chromatin foci associated with the nucleolar periphery
- Stage III: partial rim of chromatin staining at the nucleolar surface
- Stage IV: complete layer of chromatin staining, enveloping the nucleolus

The changes in GV morphology coincide with modifications in the organization of cytoplasmic microtubules (CMT) (Mattson and Albertini, 1990).

- Stage I GV: extensive CMT complex, uniformly distributed throughout the ooplasm
- Stage II GV: CMT at GV margins, extend into subcortical cytoplasm
- Stage III GV: decreased number of CMT with decreased length, in perinuclear position
- Stage IV GV: reduction in number of CMT, localization to perinuclear position, associated with the nucleolus, at the edge of oocyte.

At Diakinesis, few MTs are associated with the chromatin at the borders of the GV, followed by the appearance of a microtubule-organizing center (MTOC), with the continuing condensation of the chromatin. At prometaphase of meiosis I, many MT's are associated with the chromosomes at spindle assembly. Changes in MT organization correlate with chromatin alterations during meiotic prophase (I-IV) and the nuclear events associated with GVBD and meiotic maturation in vitro. The acquisition and expression of meiotic competence in culture requires the transition to stage III or IV GV (Mattson and Albertini, 1990).

Data on human oocytes suggests that they may follow the same pattern of centrosome (MTOC) recruitment because numerous MT nucleation sites are visible adjacent to the GV. Astral arrays of MTs can be observed throughout the cortex and in accord with reports of MTOC disposition in animal models (Messinger and Albertini, 1991), MTOCs exist in the oocytes cortex during various stages of maturation, but are most prevalent during meiotic metaphase (Battaglia *et al.*, 1996b).

At GVBD, the nuclear membrane breaks down; this is followed by condensation of dictyate chromatin into compact bivalents, formation of the first metaphase spindle and progression through meiosis I to telophase and first pb extrusion, with subsequent immediate arrest at metaphase II, the stage of maturity in human oocytes (Wassarman *et al.*, 1976). During this process of maturation, cortical granules (CG) are accumulated and migrate to the oocyte periphery, forming a protection mechanism against polyspermy; CGs are sparse and discontinuous in immature oocytes (Veeck, 1991).

Chromosomal abnormalities and abnormal embryonic development are the major causes of preimplantation embryonic death, implantation failure or spontaneous abortions. These abnormalities may arise not only after fertilization but even earlier during meiotic maturation of human oocytes (Plachot *et al.*, 1988). Meiosis occurs in two parts; in meiosis I the homologous chromosomes pair in a process of synapsis, and exchange of genetic material, known as recombination takes place between the homologous pairs. During the separation of the homologous chromosomes, the points of interchange remain united and the chromosomal structure locally has then an X appearance, known as a chiasma. The bivalents line up on the spindle, and at anaphase the two duplicated homologues separate and move to opposite poles. Each daughter cell contains two copies of one of the two homologues when the meiotic cell divides. This is followed by segregation of the homologues into the daughter cells, reducing the number of chromosomes from the diploid (2n) to the haploid (n) number (Eppig, 1996). In oocytes, the allocation of cytoplasm is asymmetric, resulting in a polar body whose size is a small fraction of that of the oocyte. This occurs because the spindle takes a peripheral location. Its positioning is tightly controlled by the cytoskeleton.

Meiotic recombination (crossing over) not only generates genetic variation but also ensures the correct segregation (disjunction) of homologous chromosomes at the first meiotic division. Important chromosomal abnormalities may arise in oocytes during meiosis if abnormal segregation of chromosomes (non-disjunction) or chromosome loss (anaphase lag) results in monosomy or trisomy in the zygote (Wramsby, 1988). The frequency and distribution of chiasmata are of major importance in the aetiology of trisomy formation (Angell, 1995). When the meiotic process occurs abnormally and homologues fail to separate at the first or second meiotic division, some of the haploid cells produced lack one or more chromosomes, while others have more than one copy. Such abnormal gametes form abnormal embryos, most of which are non-viable. However, a small proportion does survive, for example: an extra copy of chromosome 21 as a result of non-disjunction during meiotic division I or II results in Downs's syndrome (trisomy 21) in humans (Alberts *et al.*, 1994). Only chromosomes 13, 18, 21 X and Y aneuploidies can survive to birth and beyond, i.e. the sex chromosomes and the three smallest autosomes. Others die at different stages, e.g.

early or late miscarriages, pre-implantation and pre-blastocyst embryonic development.

The existence of chiasmata at MI and their location along the chromosomes is believed to relate to the process of chromosome disjunction during oocyte maturation. Currently, information on female recombination is derived indirectly from genetic linkage studies, which have implicated certain crossover configurations as a risk factor for maternal chromosome non-disjunction (Hassold and Hunt, 2001). The ability to observe them directly at this stage in in vitro matured oocytes, while difficult, would be advantageous for studies on the mechanism of origin of aneuploidies.

Prior to 1966, when Tarkowski developed the classic air-drying method for chromosome preparations from mouse oocytes, the squash method was routinely employed to study meiotic chromosomes of oocytes from several species (Edwards, 1962, Jagiello, 1965). However, oocyte squashing does not produce uniform and consistently good preparations and optimal spreading of chromosomes does not result (Tarkowski, 1966). The air-dry technique for preparing human oocytes for chromosomal analysis by light microscopy (Tarkowski, 1966) allows the visualization of normal chromosomal configurations and has allowed the identification of oocytes that exhibit either degenerate or aberrant meiotic forms (Racowsky *et al.,* 1992a,b). Racowsky *et al.* (1992a) studied nuclear degeneration and meiotic aberrations in human oocytes matured in vitro. Their findings revealed that the majority of meiotic aberrations occurred either at metaphase I or at some stage subsequent to alignment of homologues on the first metaphase plate. These aberrations fell into two classes of meiotic configuration: the clumped MI, characterized by adhesion of the chromosomes into a compacted mass, and the two groups of bivalents.

At the time of ovulation, mammalian oocytes are arrested at metaphase of the second meiotic division (MII) with the spindle composed of MT's to which maternal chromosomes are attached by their centromeres. These microtubules are involved in the separation of sister chromatids at the second meiotic division (Baka *et al.,* 1995). The MT's and associated kinetochores align the chromosomes along the metaphase

plate, and paired kinetochores on each chromosome separate allowing each chromatid to be pulled towards opposite spindle poles. The presence of an intact second meiotic spindle is a requirement for normal segregation of chromatids during the completion of meiosis. In humans, the spindle is orientated perpendicularly to the cell surface (Battaglia *et al.*, 1996a). Human meiotic spindles are symmetrical, barrel-shaped and contain anastral broad poles. Microfilament areas overlie the meiotic spindle of the human oocyte and are possibly related to normal pb extrusion. Spindle damage, caused for example by maternal aging (Battaglia *et al.*, 1996a), short-term exposure to temperatures below 37°C (Pickering *et al.*, 1990) and cryopreservation (Pickering and Johnson, 1987; Sathananthan *et al.*, 1988) can result in non-disjunction of chromatids resulting in aneuploidy (Baka *et al.*, 1995).

In 1999, Hegele-Hartung *et al.* investigated the influence of FF-MAS on chromosome, microtubule and ultrastructural dynamics in hypoxanthine-arrested mouse oocytes in a set of time course experiments. Hypoxanthine is an inhibitor of meiotic maturation; Grondahl *et al.* (1998) suggest that it may inhibit cAMP phosphodiesterase, thus maintaining a high intracellular cAMP level. Grondahl *et al.* (1998) demonstrated the ability of synthetic FF-MAS to mediate the resumption of meiosis in vitro in a dose dependent manner, in both naked and cumulus-enclosed mouse oocytes arrested in meiosis with hypoxanthine, isobutylmethylxanthine (IBMX) or dibutyryl cAMP (dbcAMP). Hegele-Hartung *et al.* (1999) found FF-MAS treated oocytes to behave similarly to spontaneously matured oocytes. Chromosomes became aligned, a barrel-shaped spindle formed and overall organelle distribution was normal. There were an increased number of small cytoplasmic asters and an increased distance between cortical granules (CGs) and the oolemma in FF-MAS treated oocytes. The short fertilizable life span of mammalian oocytes, especially in vitro is linked with the proper development of CGs during meiotic maturation; CGs are important indicators of cytoplasmic maturation. During meiotic maturation, CGs migrate to the periphery of the oocyte and remain under the oolemma until fertilization. CG loss starts at MI (~30% loss) and continues at MII in both in vitro and in vivo matured mouse oocytes. FF-MAS leads to a slow movement of CGs towards the oolemma as well as to an increase in reuptake of CG material from the perivitelline space (Hegele-Hartung *et al.*, 1999), they suggested that FF-MAS can

reduce the spontaneous CG release, observed in vitro and in vivo conditions in mouse oocytes in the absence of sperm penetration (Okada *et al.*, 1993), thereby preventing precocious modification of the zona pellucida, e.g., zona hardening (De Felici and Siracusa, 1982). These observations led Hegele-Hartung *et al.* (1999) to conclude that FF-MAS appears to improve mouse IVM oocyte quality by supporting microtubule assembly and by delaying spontaneous CG release, which is known to contribute to reduced fertilization.

In 2000, Nogueira *et al.* analyzed the nuclear status and chromosome composition of embryos derived from in vitro matured oocytes, retrieved from stimulated patients undergoing IVF. They demonstrated a similar fertilization rate of in vitro matured oocytes and in vivo matured oocytes, but a higher incidence of non-cleavage in the former along with chromosomal anomalies in 78.5% (11/14) this was associated with a high proportion of multinucleation. Multinucleation could result from nuclear disintegration during interphase or karyokinesis in the absence of cytokinesis. Nogueira *et al.* speculated that the high incidence of genetically abnormal embryos (showing aneuploidy and multinucleation) derived from in-vitro matured oocytes was due to a deficiency in cytoplasmic maturation that cannot be recovered completely by the sperm and probably causes spindle defects at cleavage.

Reduced competence for the human oocyte has been associated with spindle malformations and chromosome malalignment (Battaglia *et al.*, 1996a; Battaglia and Miller, 1997; Van Blerkom and Davies, 2001). The normal assembly of MTs and microfilaments in unfertilized oocytes is required for successful fertilization (Kim *et al.*, 1997), both are associated with the reconstruction and proper positioning of chromatin after GVBD and during meiotic maturation in human oocytes. Defects of the microtubular system can probably induce loss or gain of a single or a few chromosomes, leading to aneuploidy (Kim *et al.*, 1998). In improperly matured oocytes, chromatin is located outside of microfilament-rich areas, which seem to be related to abnormal embryonic development following fertilization (Kim *et al.*, 1998). Suboptimal culture conditions during maturation may cause insufficient maturation of cytoplasmic organelles, such as MTs and microfilaments, resulting in abnormal spindles and developmental arrest. Moreover, during IVM, the timing of phases of

meiosis might be altered, resulting in MT irregularities and unusual chromosome placement.

Goudet *et al.* (1997) studied metaphase spindles in equine IVM oocytes and in vivo matured oocytes. They found spindles obtained after IVM to be significantly wider and longer than spindles obtained at recovery of in vivo matured oocytes. A general change in the critical concentration of tubulin in the cytoplasm may induce an alteration in the size of the spindle (Eichenlaub-Ritter *et al.*, 1986). Thus it is possible that in vitro culture may allow the same nuclear maturation as in vivo maturation but may induce a change in the concentrations of cytoplasmic proteins, such as tubulin, and also other factors involved in cell cycle regulation (Goudet *et al.*, 1997), which might jeopardize the developmental competence of the resulting oocyte.

Temperature control during IVM is of paramount importance, as spindle MTs are highly thermosensitive; even a small change in temperature can disturb the spindle structure of the oocyte. In 1990, Pickering *et al.* observed disassembled spindles in 50% of oocytes after 10 min at RT and in 100% after 30 min at RT; chromosome anomalies resulted. Wang *et al.* (2001) demonstrated that a reduction in temperature to 33°C resulted in the depolymerization of spindles within 10 min.

In view of the promising results obtained with FF-MAS in promoting the survival and maturation of human GV oocytes (Chapter 5) and similar data in animals from other groups, the maturing oocytes' chromosomal constitution and spindle appearance was studied to determine whether the oocytes in which FF-MAS had stimulated maturation were developing normally. This assessment was undertaken in oocytes from patients undergoing ICSI treatment only, this being the more readily available source of human oocytes, and potentially less likely to have anomalies already present than oocytes from patients with PCO whose follicular environment may be compromised.

6.2 Aims

To assess the feasibility of studying human oocyte chromosomes and chiasmata at metaphase I during IVM. To attempt IVM of oocytes obtained from patients undergoing a cycle of ICSI treatment, in conditions including FF-MAS at a concentration of 30μg/ml or control (no FF-MAS, vehicle only). To compare in vitro matured with in vivo matured (IVF failed fertilized) oocytes, in terms of spindle and chromatin organization. To observe differences in the cytology and chromatin of oocytes maturing or remaining immature under control or FF-MAS conditions.

6.3 Chromosome assessments of MI oocytes from patients undergoing a cycle of ICSI treatment

6.3.1 Patients

For this preliminary study, a total of 19 immature oocytes were collected from eight patients undergoing cycles of ICSI treatment. Clinical details of the patients are presented in table 3.3 (chapter 3).

6.3.2 Oocytes

The immature oocytes were cultured with colcemid (10μg/ml) for 4-6 hr at 37°C, 5% CO_2 in air before fixing and spreading (Chapter 2, section 2.6).

6.3.3 Results from chromosome spreading

Four of the 19 oocytes did not progress beyond the GV stage, 14 displayed no pb and were presumed to be in MI and one was in MII at the time of spreading. Only three analysable spreads were obtained from 15 oocytes studied. In one of these, 22 bivalents and several chiasmata were evident (Figure 6.1). In the MII oocyte, 14 chromosomes were observed in the oocyte and five in the pb. In the third preparation, only five chromosomes were seen.

In view of the disappointing results obtained, despite supervision by an experienced cytogeneticist, this line of investigation was discontinued.

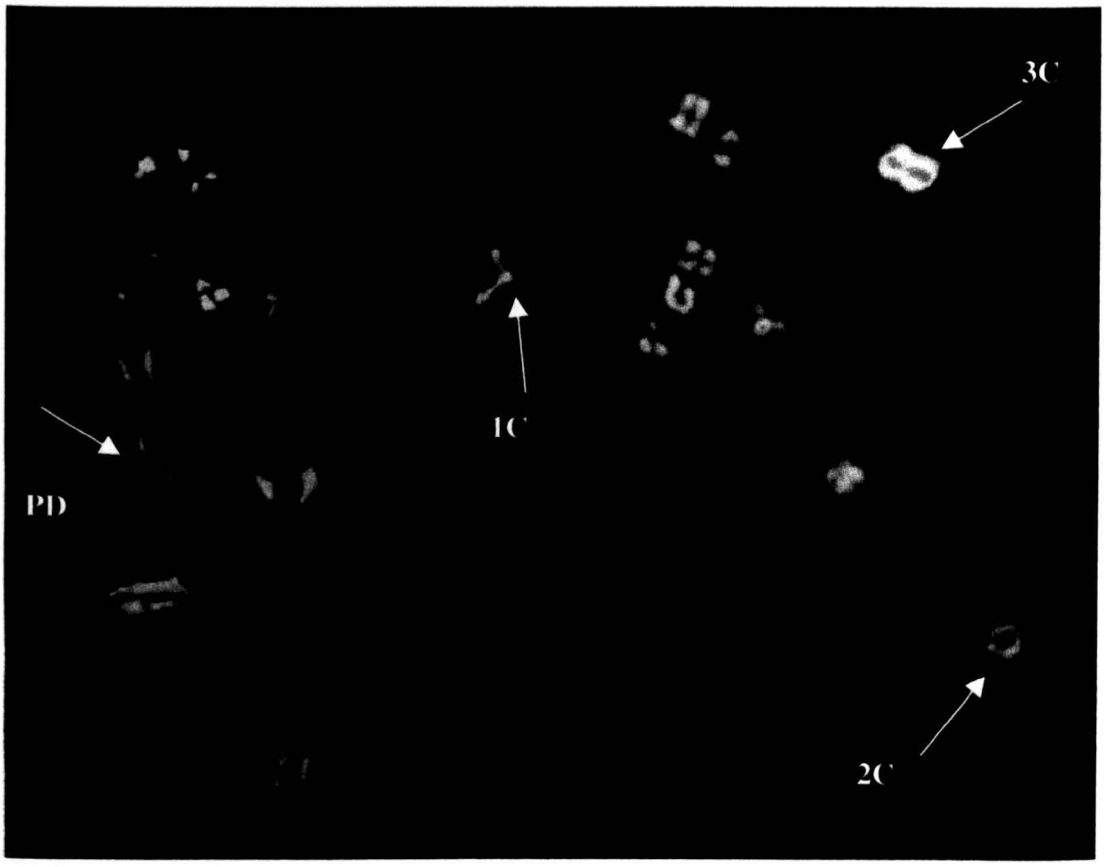


Figure 6.1 Spread of chromosomes from a human oocyte at first meiotic metaphase, during maturation in vitro.
22 chromosomes are evident. One shows signs of three chiasmata (3C), whereas several show signs of two chiasmata each (2C) or one chiasma (1C). Premature dissociation of chromosomes pairs in the left hand group is apparent (PD). The identification of individual chromosomes is not possible for a spread of this quality.

6.4 Effect of FF-MAS on spindle formation in in-vitro maturing oocytes from patients undergoing a cycle of ICSI treatment

6.4.1 Patients

For this study, a total of 35 immature oocytes were collected from 21 patients undergoing cycles of ICSI. Clinical details of the patients are presented in table 3.3 (chapter 3).

6.4.2 Oocytes

The oocytes were cultured with 30μg/ml FF-MAS, the concentration shown in previous experiments to promote IVM in this source of oocytes, or with no FF-MAS (control) for 24 hr; spindle analysis as described in section 2.7.1 was then performed on mature oocytes.

Oocytes were considered mature if a polar body was visible by Hoffman microscopy. Oocytes failing to mature were stained with Hoechst 33258 to confirm their chromatin status as described in 2.5.3.

6.4.3 Spindle analysis

The position and shape of the MII spindle and the normality of chromosomal alignment in oocytes were assessed using fluorescence microscopy after preparation of oocytes by staining with anti-tubulin antibodies and DAPI, as described in section 2.7.1

6.4.3.1 Mouse oocytes

Preliminary work carried out using mouse oocytes demonstrated the reliability of the technique and was used to confirm appropriate antibody concentrations. Figure 6.2 shows the normal equatorial alignment of chromosomes in a mouse oocyte at MII after staining with antitubulin antibodies and DAPI. The polar body had degenerated.

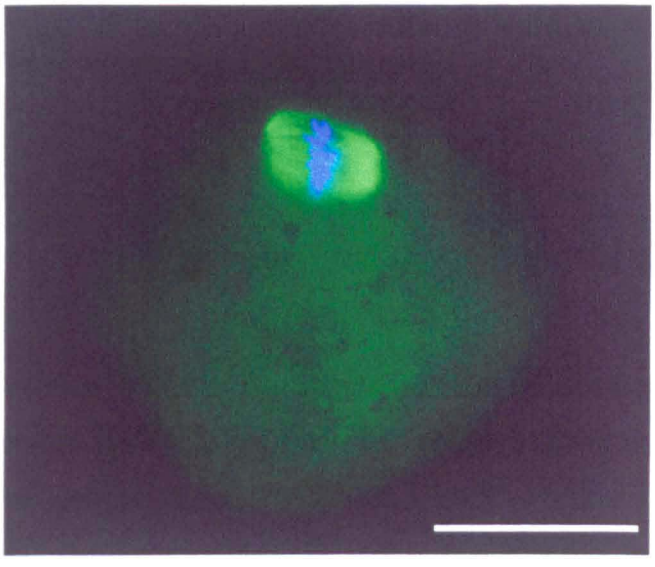


Figure 6.2 Mouse oocyte stained with FITC-labelled anti-tubulin antibodies (green) and DAPI (blue). The position of the spindle (intense green) parallel with the oocyte surface is characteristic in mice. The chromosomes appear blue and are arranged in a compact line across the spindle indicative of the normal arrangement.

Bar = 25 μm

6.4.3.2 Human oocytes

Human oocytes were randomized to either 30μg/ml FF-MAS or control conditions, 15 oocytes were cultured in 30μg/ml FF-MAS and 12 were considered to have matured, as shown by the presence of a pb. One oocyte arrested at the GV stage, one remained immature and one oocyte became atretic in culture.

20 oocytes were cultured in control conditions, three oocytes matured, five arrested at the GV stage, seven remained immature (six oocytes arrested at GVBD and one oocyte at MI) and five degenerated in culture. Three oocytes (MII and GVBD or MI in control and MII in 30μg/ml FF-MAS) could not be analysed after culture due to unforeseen circumstances. The results presented in Table 6.1 confirm those in Chapter 5, that 30μg/ml FF-MAS significantly promotes maturation of oocytes (from patients undergoing ICSI treatment) in vitro ($p<0.001$).

Table 6.1 Outcome of in vitro culture with or without FF-MAS of oocytes donated by patients undergoing ICSI treatment.				
	Mature	Immature	Atretic	Total
Control	3	12	5	20
FF-MAS (30µg/ml)	12	2	1	15

Oocytes were identified as having nucleolar chromatin condensation corresponding to Mattson and Albertini (1990) stages II to IV. Table 6.2 shows the chromatin staging of all non-matured oocytes stained with Hoechst 33258. For the oocytes cultured with FF-MAS (30µg/ml) the oocyte with a GV visible was observed to be at stage III with a partial rim of chromatin staining at the nucleolar surface. For the oocytes cultured in control, there were five with visible GVs, four of these were stage II GVs and one was stage III. For the seven remaining immature oocytes, in which no GV was seen, one had stage III chromatin, three had stage IV chromatin, one was at MI and two were not examined. Oocytes with a GV visible at the time of staining had stage II or III chromatin.

Table 6.2 Chromatin staging of non-matured oocytes, donated by patients undergoing ICSI treatment and cultured with or without FF-MAS.							
	GV			**MI**	**Non-identifiable/not examined**	**Atretic**	**Total**
	II	III	IV				
Control	4	2	3	1	2	5	17
FF-MAS (30µg/ml)		1		1		1	3

Figure 6.3a,b and c shows hoechst images of oocytes that arrested at the GV stage after culture in control.

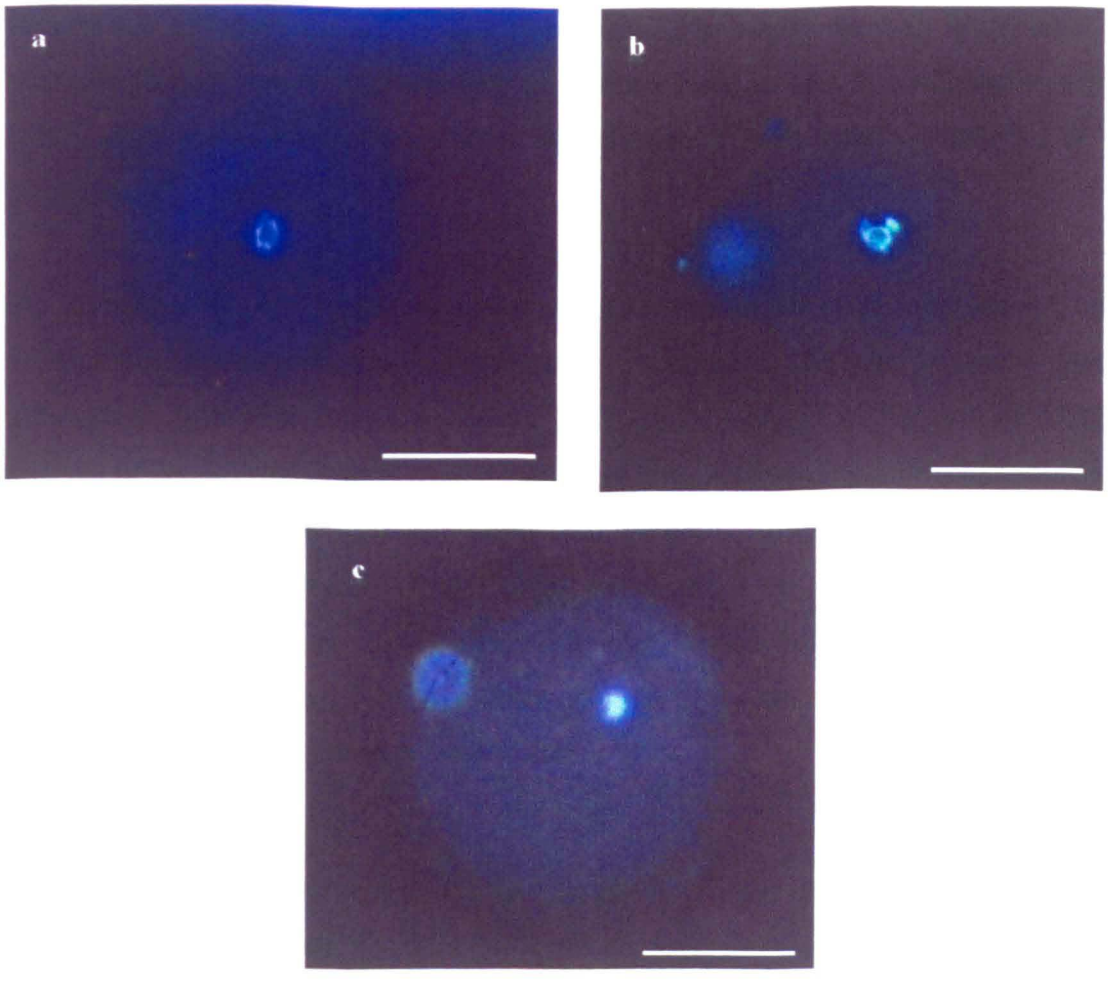


Figure 6.3 Nuclear staining with Hoechst 33258 of human oocytes that arrested at the GV stage after culture in control conditions.
a) Stage II GV, 2-3 chromatin foci associated with the nucleolar periphery (Mattson and Albertini, 1990).
b) Stage III GV, partial rim of chromatin staining associated with the nucleolar periphery.
c) Stage IV GV, complete layer of chromatin staining enveloping the nucleolus

Bar = 50μm

Figures 6.4- 6.6 show the results of spindle staining in human oocytes. When tubulin was identified immunocytochemically in oocytes fixed at MII in combination with DAPI staining of chromatin, spindles and chromatin were readily identified in a proportion of oocytes.

No spindles were observed in the two oocytes matured under control conditions, one for technical reasons (oocyte damaged during preparation) the other oocyte stained, although no spindle was seen. Of the 12 MII oocytes matured in 30μg/ml FF-MAS, five out of six oocytes stained were observed to have abnormal spindle structures and the sixth oocyte was not analysed (see p.136). Figure 6.4 shows a spindle with some displacement of chromatids of an oocyte at anaphase of MII. Figure 6.5 shows examples of abnormal spindles. Technical failure affected five oocytes; three oocytes failed to stain (nothing seen on slide) and two oocytes crumpled during slide preparation making observation impossible.

Eight in vivo matured oocytes that had failed to fertilize after clinical ICSI were stained using the same protocol, in order to act as controls. Two oocytes exhibited normal spindle structures (an example is shown in figure 6.6) although poor alignment of chromosomes was evident, possibly due to in vitro aging known to occur (Asch *et al.*, 1995). Three oocytes exhibited abnormal spindle structures and the three remaining oocytes failed to stain.

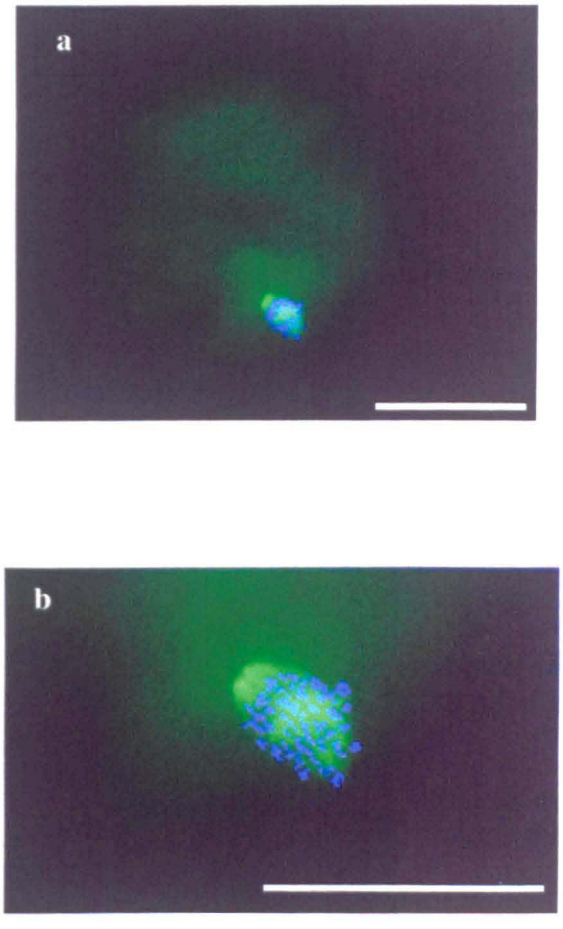
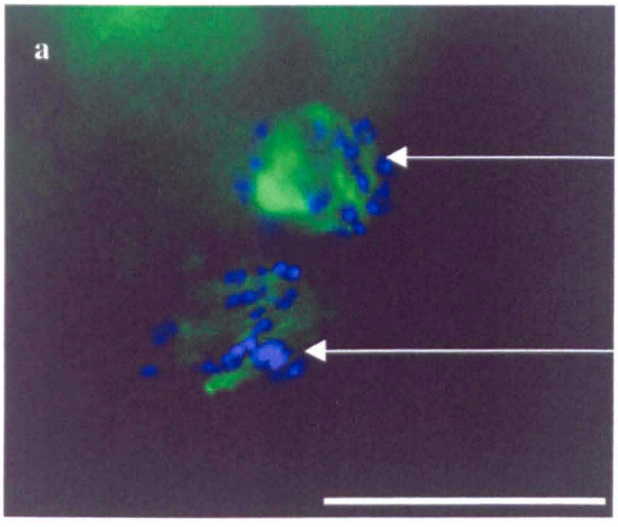


Figure 6.4 Combined DAPI and FITC fluorescence in a mature human oocyte cultured in 30µg/ml FF-MAS, at anaphase of MII. The spindle can be seen, with the chromatids separating and moving to opposite poles. Some chromatids are displaced from the spindle.

Bar = 50 µm



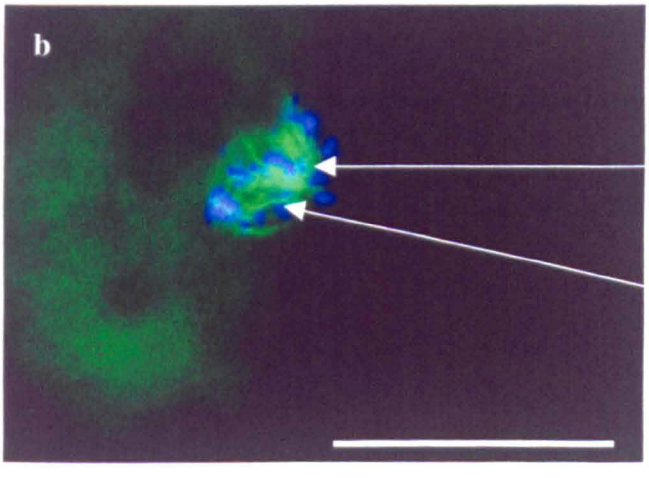


Figure 6.5 Examples of abnormal spindles in three human oocytes matured in vitro in the presence of 30µg/ml FF-MAS.
a) A disorganized spindle is present towards the periphery of the oocyte, adjacent to the polar body. The chromosomes are not aligned normally on the oocyte spindle. Chromosome disorganization within the first polar body is common and does not affect the genetic complement of the oocyte.
b) A disorganized spindle with dispersed chromosomes is evident. This was observed in the centre of the oocyte. The polar body is not visible in this image.

Bar = 50 µm

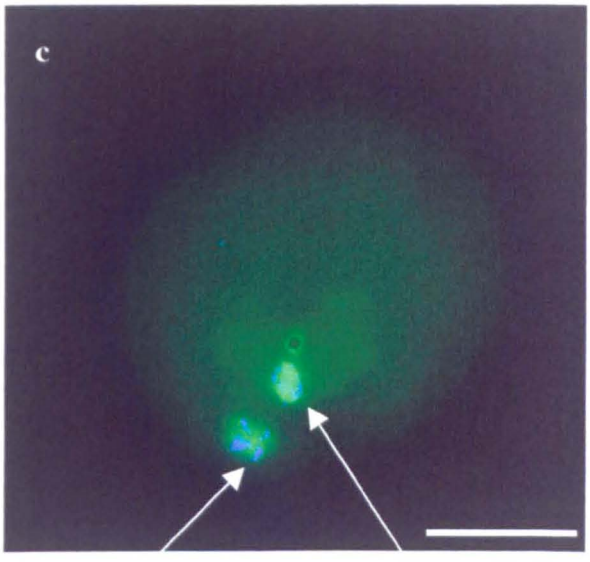


Figure 6.5 Examples of abnormal spindles in three human oocytes matured in vitro in the presence of 30µg/ml FF-MAS.
c) A small spindle is evident towards the periphery of the oocyte with some chromosomes aligned at the midpoint and others mal-aligned. The polar body is in the process of division.

Bar = 50µm

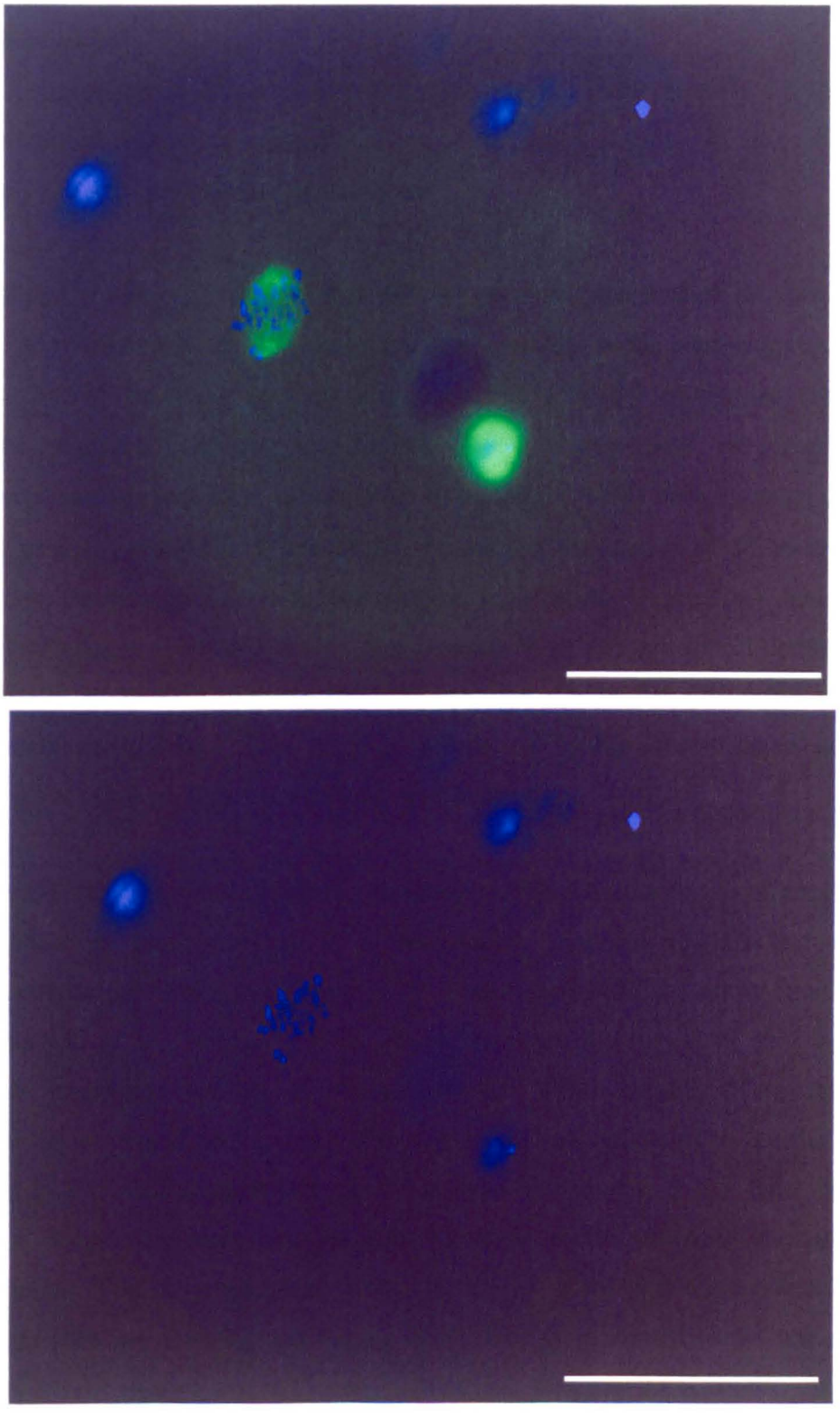


Figure 6.6 Example of a normal shaped spindle in an oocyte that failed to fertilize. Individual spindle fibres visible and chromosomes in pairs but dispersed on the spindle. The other spindle (not in focus, is the polar body), the clear area in the centre of the oocyte is a vacuole, sperm and/or degenerating cumulus cells can be seen around the edge of the oocyte. Bar = 50μm

6.5 Discussion

In this chapter, three different approaches have been used to identify whether meiotic progression is occurring normally in vitro; chromatin staining in situ, chromosome spreads and spindle structure assessments.

Chromatin staining of oocytes that did not complete maturation in vitro showed a range of arrest points, and this was particularly evident in the control group where the proportion of maturing oocytes was low. The classical chromatin condensation patterns observed by Mattson and Albertini (1990) were evident, and clearly, some of the immature oocytes were incompetent to undergo GVBD under control conditions, being at stages II and III. Interestingly, the oocytes exposed to FF-MAS had matured in a significantly ($p<0.001$) higher proportion, confirming previous data, and they appeared to have overcome the GV arrest observed in a proportion of control oocytes, potentially suggesting that the FF-MAS can act during the GV stage to promote chromatin maturation. This point is worthy of further investigation with larger numbers of oocytes, if this can be achieved in humans.

Colcemid is a microtubule inhibitor, therefore nuclear progression is blocked at MI by prevention of the formation of a MI-spindle, however GVBD and chromatin condensation take place in an apparently normal manner in the presence of colcemid (Wassarman *et al.*, 1976). In 1995, Angell reported the results of first and second meiotic metaphase spreads of human oocytes. While spreads of second meiotic metaphase were obtained in some 70% of oocytes that had failed to fertilize or cleave during IVF cycles (Angell, 1995), MI oocytes are known to be more difficult to prepare. MI oocytes were obtained from volunteer women scheduled for laparoscopic sterilization. The MI preparations lacked the clarity of MI preparations from mice and accurate chiasma counts could not be made. However, Angell (1995) reported on a total of 22 oocytes, of which 18 had 23 bivalents, two had 22 bivalents with one pair of homologous univalents, and two at early anaphase, had two pairs of homologous univalents.

The results of my attempts to obtain interpretable MI spreads were disappointing including loss of chromosomes, probably due to excessive spreading. The one useful spread showed 22 chromosomes. One chromosome showed signs of three chiasmata whereas several showed signs of two chiasmata each or one chiasma. Premature dissociation of chromosome pairs in the left hand group was apparent. The identification of individual chromosomes was not possible.

MI occurs during the period of IVM and therefore it was important to this study to attempt to visualize chiasmata at MI. As crossing over occurs in fetal life at prophase I, long before IVM, observations from in vitro matured oocytes would be unlikely to differ from standard maturation, and would add greatly to the currently limited knowledge base on this topic.

An alternative approach to analysing crossing over uses antibody labeling to MLH1, the DNA mismatch repair gene. MLH-1 forms foci along the axes of paired homologous chromosomes in germ cells at prophase I of meiosis. In 1998, Barlow and Hultén analyzed crossing over at pachytene in human spermatocytes with anti-MLH1 antibody labeling, as in the mouse the numbers and distributions of MLH1 foci matched those of chiasmata at MI. Had visible spreads been obtained, it had been hoped to apply anti MLH1 antibody to identify chiasmata. However, a recent study by Tease *et al.* (in press), which detected MLH1 foci in human fetal oocytes, showed that the foci remained clear on synaptonemal complexes from zygotene to pachytene but started to disappear in early diplotene, and so would most probably have been undetectable in MI oocytes. This assessment was therefore not undertaken in my study.

Even using the up to date microscopy and cytogenetic technology now available, chiasmata remain difficult to visualize. This pilot study has confirmed that the spreading technique is technically difficult, in view of the limited supply of human oocytes and the lack of analysable results obtained it was decided that an assessment of mature oocytes at MII would be more productive.

My results (Chapter 5 and Chapter 6) showed significant effects of FF-MAS on human oocyte maturation in vitro. However, the manner in which FF-MAS promotes maturation is uncertain and the normality of maturation under in vitro conditions required further exploration. This would be critical for any future application in therapeutic regimes. This preliminary study therefore aimed to examine the spindle organization in morphologically normal oocytes, arising after IVM with or without culture with FF-MAS. The incidence of maturation in vitro in the presence of FF-MAS was significantly greater than that in its absence (confirming previous results in chapter 5), however, this resulted in a relative shortage of oocytes maturing without FF-MAS exposure, limiting the interpretation of the results. However, the data show that spindle malformation affected a high proportion of oocytes matured in vitro with FF-MAS (30μg/ml). Five out of six MII oocytes with stained spindle structures were abnormal. It cannot be determined from the data whether this was due to the maturation in vitro or the FF-MAS since control oocytes rarely matured in vitro and two of those which appeared to mature were not analysed with the third having no spindle structure visible.

The normal MII spindle structure is a symmetrical barrel-shaped structure located eccentrically, with the 23 chromosomes each with two sister chromatids, aligned on the equatorial or metaphase plate. Spindle microtubules attach to the centromeres. The chromosomes are divided between the oocyte and the pb (23 chromosomes, 46 chromatids, 2n DNA in each). The first pb remains connected to the oocyte by a cytoplasmic bridge for a while after its formation. The chromosomes within the first pb may remain clumped together, undergo a second meiotic division or scatter within its cytoplasm (Veeck, 1999).

In Figure 6.4, the spindle was symmetrical and barrel shaped with correct positioning, the oocyte appeared to be at anaphase as the chromatids had split at the centromeres and had begun to move to opposite ends of the spindle apparatus. As the oocyte had not been fertilized, chemical exposure or environmental trauma may have activated the oocyte causing the resumption of meiosis. Some chromatids can be seen detached from the spindle apparatus, although scattering throughout the cytoplasm was not evident.

Figure 6.5 shows oocytes exhibiting spindle defects; these oocytes were also characterized by the occurrence of detached chromosomes. In Figure 6.5a, the spindle is disorganized, not barrel shaped, rounded instead of pointed at the poles and the chromosomes are displaced. In Figure 6.5b, the spindle is not located in an eccentric position and again, the chromosomes are mal-aligned, however they are not scattered throughout the cytoplasm.

Baka *et al.* (1995) saw some dispersion of chromosomes throughout the cytoplasm in prophase I human oocytes which had matured in vitro, however 83.8% of the oocytes showed a normal appearance of the meiotic spindle and chromosomes. Tremoleda *et al.* (2001) studied the organization of the cytoskeleton during IVM of horse oocytes. Both the microtubular and microfilament elements of the cytoskeleton were seen to reorganize in a fashion that appeared to enable chromosomal alignment and segregation. In Figure 6.5c, the spindle is in the correct position located at the periphery of the cell and orientated radially with its long axis perpendicular to the surface membrane but it is disorganized with chromosome malalignment.

Similar to the study by Hegele-Hartung *et al.* (1999) analyzing the effects of FF-MAS (10μg/ml) on the microtubular cytoskeletal and chromosomal organization in mouse oocytes via double-labeling fluorescence; the majority of spindles of MII oocytes were barrel shaped with a dense network of microtubules appearing to fill the region between the poles and the chromosomes. However, Hegele-Hartung *et al.* (1999) observed chromosome alignment in FF-MAS matured oocytes, which was disrupted in most of the oocytes maturing in culture with FF-MAS in this study.

In 1996, Battaglia *et al.* studied the influence of maternal ageing on meiotic spindle assembly in oocytes from naturally cycling women. They found that ~80% of oocytes aspirated from antral follicles of naturally cycling women between 40-45 years contained abnormally organized spindles with malaligned or detached chromosomes In contrast, only 17% of oocytes from antral follicles of naturally cycling women between 20-25 years exhibited aneuploid conditions (Battaglia *et al.*, 1996a). Battaglia *et al.* (1996a) studied the various phases of meiosis; they observed condensed chromosomes throughout GVBD (as expected), which became organized

as meiosis progressed, they were unable to visualize the MI spindle and concluded that, similar to animal models, the first metaphase spindle was transient. At MII, a distinct spindle was observed with chromosomes aligned on the metaphase plate.

Aged oocytes display an increased incidence of disrupted or abnormal cytoskeletal organization (Eichenlaub-Ritter *et al.,* 1988; Pickering *et al.,* 1988). Poor alignment of chromosomes was evident in oocytes that had failed to fertilize, probably due to in vitro ageing (Asch *et al.,* 1995). The patient group used in my study was aged between 25-40, with a mean age of 33.8 years; it is therefore unlikely that increased maternal age was a major factor for most of the abnormal spindles observed here.

Microtubules of the metaphase spindle are highly thermo-sensitive. Even a small change in temperature can disturb the spindle structure of the oocyte (Munné and Cohen, 1998). Human oocytes have exhibited a disassembled spindle in 50% of cases after 10 min at room temperature (RT), and in 100% after 30 min at RT accompanied by chromosome abnormalities (Pickering *et al.,* 1990). In my study, cooling was not a causative agent in the abnormal spindles observed, as all oocytes were fixed. The fix used was formaldehyde, although this is not a fast fix, the relatively small size of the oocyte helps to ensure fast fixation.

This preliminary study of spindle normality after IVM of human oocytes with FF-MAS suggests that there are major abnormalities in the chromosomes distribution upon the oocytes' spindles after IVM. The cause of this is uncertain, potentially relating to either exposure to FF-MAS, or the IVM process or conditions, but difficulties in establishing an adequate control group of oocytes prohibit further interpretation. The gross abnormalities in spindle development may be a major contribution to the limited developmental potential of oocytes matured in vitro observed in this series and reported by several other groups. However, the number of studies which have examined this issue remain small and thus conclusions are not yet available. It will be crucial to follow up the possibility of abnormal chromosome complements in in vitro matured oocytes, since the normality of any offspring as well as success rates may depend upon this. However, even when chromosomally normal oocytes are formed, the normality of the cytoplasmic component remains uncertain.

Future work on the genetic results of in vitro matured oocytes in the presence of various factors known to stimulate maturation is required, however, there is a major need to develop non-invasive measures of developmental competence which could be applied in the clinical situation. The next chapter explains some of the measurable morphological features which might contribute to the development of such non-invasive method

Chapter 7

Image analysis

7.1 Introduction

Oocyte IVM may result in viable mature oocytes and in some respects may mimic the in vivo process (Schroeder and Eppig, 1984). However, all oocytes within the ovary are not equally competent to resume meiosis in vitro and reach full maturity. Oocyte competence to undergo GVBD and mature to MII is primarily determined by the developmental stage of oocyte, including its volume, reflecting adequate growth, and these aspects are closely coordinated and controlled by the follicular environment and stage of development (Gilchrist *et al.,* 1995). As described in the introduction (Chapter 1) the human oocyte and the follicle initially grow in parallel in a tightly regulated manner, however, beyond the point of antrum formation, oocyte growth declines markedly while follicle growth continues apace. However, the somatic and germ lines remain closely in contact and their further development is effectively symbiotic.

In 1985, Tsuji *et al.* examined the relationship between the follicular size in the human ovary and the oocytes' capability of resuming meiosis in vitro in each phase of the menstrual cycle. In the follicular phase, the percentage of immature oocytes resuming meiosis and extruding a polar body in the large-follicle group (9-15mm diameter) was significantly higher than that in the small-follicle group (3-4mm diameter) ($p < 0.05$). The capacity for meiotic maturation therefore seemed to increase with increasing follicle size. However, in the luteal phase the incidence of maturation was fairly constant, irrespective of the follicular size. Based on their results, Tsuji *et al.* suggested that the capacity for human oocyte maturation is closely related to follicular status, and the differing hormonal environments in the follicular and luteal stages, associated with differing follicular maturity may affect oocyte function.

Lonergan *et al.* (1994) studied the effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro, demonstrating that more oocytes with many layers of cumulus ($p < 0.01$) and a higher

proportion of blastocysts ($p < 0.01$) were obtained from follicles of diameter >6 mm compared to 2-6mm follicles.

In 1989, Eppig and Schroeder isolated oocytes with increasing mean diameters from juvenile mice of increasing ages. They found that oocytes from mice 13 days of age or younger, whose mean diameter was less than 60µm, were able to undergo spontaneous GVBD in culture, but an increasing percentage of larger oocytes from mice 15 days of age and older was able to undergo maturation in culture. With increasing age and mean oocyte diameter, more oocytes progressed to MII. In mice, competence to undergo GVBD was normally achieved when oocytes reached ~60µm in diameter, the capacity to progress to MII being acquired subsequently. Hirao *et al.* (1993) confirmed that this principle of increasing developmental competence with increasing size also applied to in vitro grown mouse oocytes, where the threshold diameter of 60µm for GVBD remained the same.

The capacity to cleave after maturation and insemination in vitro is also acquired with increasing age and mean oocyte diameter. Bao *et al.* (2000) demonstrated that the ability of mouse oocytes to support development occurs in a stepwise manner as oocyte diameter increases from 65-75µm. Oocytes with a diameter of 65-75µm cleave to the two-cell stage at normal rates, but subsequent developmental changes occurring in the oocytes during the final stages of the growth phase are critical for full developmental competence. Maternal chromatin is first competent to support development to term during the latter half of oocyte growth when oocytes are 60-69µm and 50-59µm in diameter in immature and adult female mice, respectively (Bao *et al.*, 2000). Thus it appears that epigenetic modifications of maternal chromatin necessary for development to term are established earlier in the oocyte growth phase in the adult ovary. This distinction between adult and juvenile ovaries is important since many studies in mice are performed on immature, relatively synchronized cohorts of follicles, not reflecting the physiological situation during reproduction.

Daniel *et al.* (1989) demonstrated an increased ability to undergo GVBD with increasing mean oocyte diameter in a rat model of in vitro follicle growth. At the start

of follicular growth in vitro, each follicle contained an oocyte in the midgrowth phase, with a mean diameter of 40-45μm, which was incapable of undergoing meiotic maturation. Oocytes that grew to full size had a mean diameter of 70-80μm. Oocytes capable of undergoing GVBD after in vitro culture were significantly larger than incompetent oocytes. No oocytes less than 55μm in diameter were GVBD competent. This indicates a relationship between oocyte growth and acquisition of meiotic competence. However, Canipari *et al.* (1984) observed mouse oocytes that became GVBD competent after being cultured in conditions that did not promote significant growth. They reported an inverse relationship between acquisition of meiotic competence in vitro and initial size of oocyte at the time of isolation, and that meiotic competence corresponded to the timing of the event in vivo. This suggests that the events of meiotic resumption and oocyte growth may be separable when non-physiological conditions are applied in vitro.

Hirao *et al.* (1994) studied the in vitro growth and maturation of pig oocytes. Progression to metaphase II was observed in 40% of oocytes that were >110μm in diameter, whereas no oocyte <90μm in diameter resumed meiosis.

Fair *et al.* (1995) studied bovine oocyte diameter in relation to maturational competence and transcriptional activity. Four groups of oocytes based on diameter were studied: <100, 100-<110, 110-<120 and >120μm. The proportions achieving in vitro development to MII were 21.2%, 42.3%, 75.9% and 80.7% respectively for the four size groups. Oocytes <110μm were shown to be actively synthesizing RNA, as demonstrated by measuring the degree of 3H-uridine incorporation in vitro and they were still in the growth phase of oocyte development. Whereas those which were larger, demonstrated a much lower degree of 3H-uridine incorporation.

The situation in primate oocytes appears to be similar. In 1993, Schramm *et al.* studied the meiotic competence of rhesus monkey oocytes. They demonstrated that the oocytes attain maximum size after antrum formation in this species, although at this stage MII competence is still low and increases with follicle diameter while oocyte size remains constant. Adachi *et al.* (1982) reported an increase in meiotic maturation of marmoset monkey oocytes with increasing follicular size, although

oocyte diameter did not change. In 1995, Gilchrist *et al.* also studied the meiotic competence of marmoset monkey oocytes in relation to follicle size and oocyte-somatic cell associations. They further demonstrated the sequential acquisition of oocyte GVBD and MII competence with increasing follicular size, however they were unable to obtain oocyte diameter data, as they did not disturb the oocyte-somatic cell contacts before culture. Equine oocytes seem to behave similarly to those of the rhesus monkey in that meiotic competence is not necessarily associated with maximum oocyte diameter, and oocytes that appear fully grown may still need to undergo cytoplasmic maturation before they are competent for optimal maturation (Goudet *et al.*, 1997). There may therefore be species differences in the point at which meiotic competence is reached, and its relationship with oocyte and follicle size.

During this study, longitudinal cultures of up to four days were performed. This afforded the opportunity to quantify human oocyte growth under the in vitro conditions employed, and to explore the possibility of using a non-invasive measure of oocyte development as an indicator of subsequent developmental competence. Such studies have not previously been published.

7.2 Oocytes from PCO patients, cultured with or without FF-MAS.

7.2.1 Oocyte diameter

The reproducibility of the image analysis measurements was assessed by a series of trial measurements performed as described in section 2.8.1. The results are shown in Table 7.1. The standard deviations observed were well within 1% of the mean value in 9 out of the 10 oocytes after measuring 10 times each, and all results were within ± 2μm of the mean, most within ± 1μm. The confidence that could be placed in these measurements was therefore high with good reproducibility.

Table 7.1 Control oocyte measurements

Oocyte	1	2	3	4	5	6	7	8	9	10
	105	103	104	103	110	107	105	108	106	110
	103	105	103	104	109	107	105	107	107	110
	104	104	105	104	110	105	106	107	105	112
	106	105	105	105	111	106	107	106	106	112
	105	105	105	104	110	106	105	106	106	109
	106	104	105	105	109	106	105	107	108	108
	106	103	104	103	109	107	106	107	106	109
	105	105	105	103	109	106	105	109	107	109
	105	104	105	105	110	107	107	108	105	110
	104	104	104	105	111	107	107	107	106	110
Mean	**104.9**	**104.2**	**105.8**	**104.1**	**109.8**	**107.2**	**105.8**	**107.2**	**106.2**	**109.9**
Std dev	**0.994**	**0.789**	**0.771**	**0.876**	**0.789**	**0.699**	**0.919**	**0.919**	**0.699**	**1.287**
Sem	**0.3**	**0.2**	**0.2**	**0.3**	**0.2**	**0.2**	**0.3**	**0.3**	**0.2**	**0.1**
Range	**3**	**2**	**2**	**2**	**2**	**2**	**2**	**3**	**3**	**4**

Table 7.2 presents the number of oocyte measurements available for each parameter measured on the day of oocyte collection and on day 0 (day of insemination of mature oocytes) for both patient groups. For patients with PCO, a total of 128 oocytes were collected (Chapter 5). Table 7.2 shows that on the day of oocyte collection, the measurement of oocyte diameter provided the largest number of measurements, a total of 86 oocytes were measured. A total of 42 oocytes were not measured on the day of collection, due mostly to a dense surrounding of cumulus cells obscuring the oolemma, making measurement impossible or unreliable. In some cases, by enhancing the image contrast and converting to grey scale it was possible to visualize and measure the oolemma through the attached cumulus cells. Fewer of the other measurement types were available because for those involving the zona pellucida, the zona required to be completely clear of any cumulus attachments and with clear visibility throughout for the measurement to be made, which only occurred for a relatively small proportion of oocytes.

For patients undergoing ICSI treatment, a total of 72 oocytes were donated, 48 oocyte diameters were measured on the day of collection and 24 were not measured. Eight oocytes were not measured on the day of collection or on day 0 due to camera failure

preventing images being taken. The rest were omitted because very faint oolemmas made measurement impossible and/or cumulus cells obscured visibility as already discussed. The cumulus cover on these immature oocytes was very difficult to remove totally using the standard hyaluronidase technique since it is well known that immature cumulus is relatively tight and dense compared to a mature expanded cumulus mass.

For all parameters measured, and for both patient groups, the number of oocytes measured on day 0 was more than or equal to the number of oocytes on the day of collection. This was due to cumulus expansion in vitro and the stripping of cumulus cells in preparation for ICSI, which enabled measurements to be made more readily.

Table 7.2 Total number of oocyte measurements available for each parameter measured on the day of oocyte collection and on day 0 (day of insemination of mature oocytes) for both patient groups.

Patients	Day	Oocyte diameter	Oocyte+zona diameter	Zona pellucida thickness	Perivitelline space (PVS)
Unstimulated	Collection	86	48	44	12
PCO	0	90	48	47	26
Stimulated	Collection	48	46	45	38
ICSI	0	61	60	59	39

Figure 7.1a presents the frequency histogram of the mean diameter of measurable viable oocytes collected from patients with PCO on the day of collection (day-2). These results approximate a normal distribution with a mean diameter of 106μm. However, the diameters on day-2 of oocytes that went on to mature in vitro irrespective of conditions of culture did not follow a normal distribution (Figure 7.1b). The data in Figure 7.1c show that the proportion of oocytes in each size range which matured showed a tendency to increase with increasing size, while no oocytes of diameter <100μm matured. Within this series, one oocyte with a mean diameter of 100μm on day-2 reached MII subsequently, which represents the minimum "threshold" observed for maturation. However, the incidence of oocyte maturation in this size range may be lower than for larger oocytes.

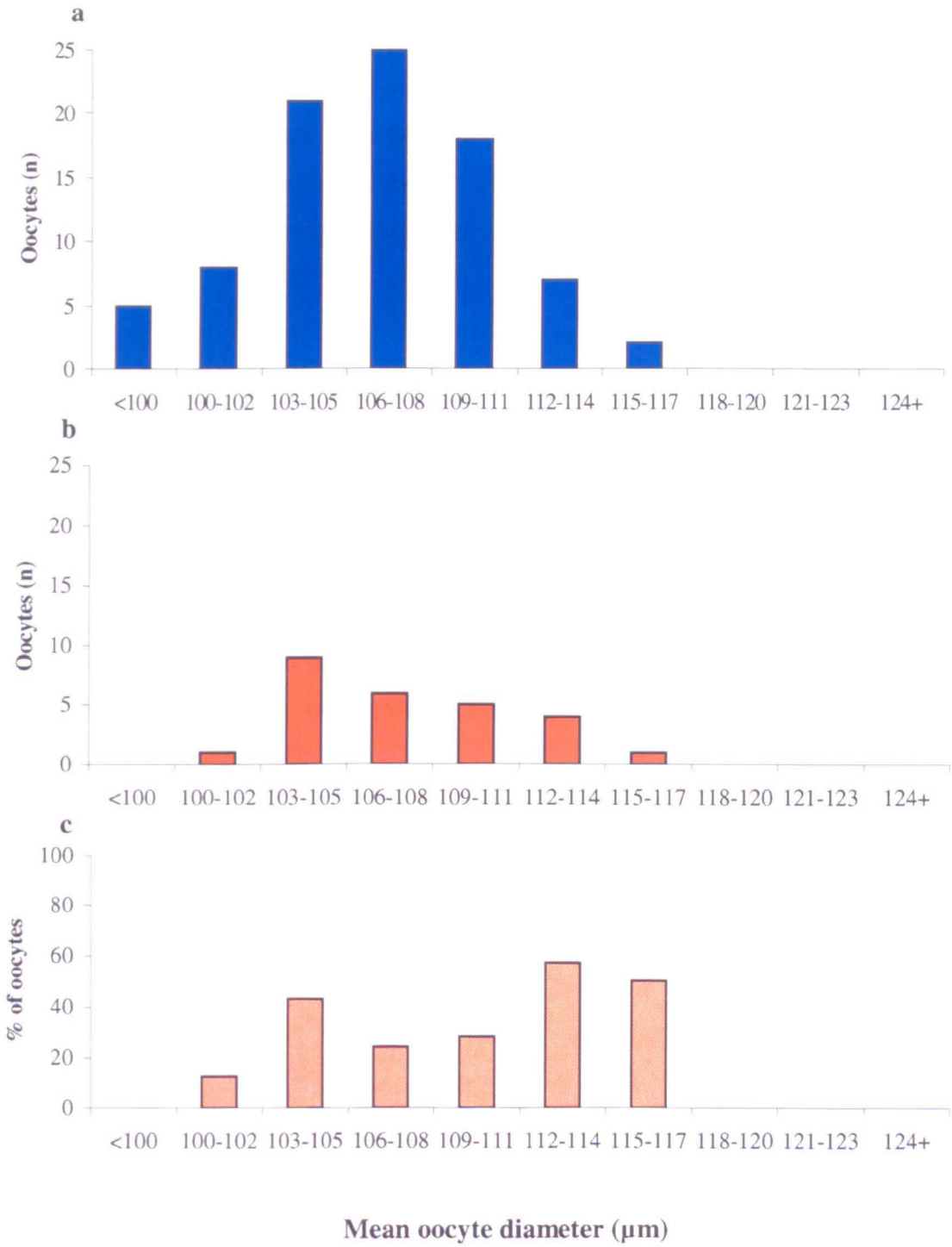


Figure 7.1 Frequency histogram showing mean oocyte diameters on day of collection (day-2) of oocytes from unstimulated patients with PCO.
a) Viable oocytes
b) Oocytes becoming mature in vitro
c) Oocytes that progressed to maturity expressed as a percentage of viable oocytes on day-2

Figure 7.2 shows the frequency histogram of oocyte diameter of oocytes from unstimulated patients with PCO on day 0 (the day of insemination of mature oocytes) according to whether the oocytes matured in vitro. More than half (55%) of the oocytes that did not mature (remained immature or became atretic) had a mean diameter of 105μm or below on day 0 (Figure 7.2a). One oocyte that remained immature measured 140μm in diameter, this abnormally large oocyte was probably diploid.

For oocytes that did mature, only 18% fell into the same size category on day 0 (Figure 7.2b). Among the mature oocytes, 43% had a diameter >109μm compared to 21% in this size range among those oocytes not maturing.

Figure 7.2c shows that the threshold for oocyte maturation in this study was 103μm on day 0, as compared with a minimum of 100μm on day-2. However, the majority of oocytes (82%) that matured had diameters on day 0 of 106μm or above. Figure 7.2c shows that the proportion of oocytes achieving maturity tends to increase with increasing diameter on day 0.

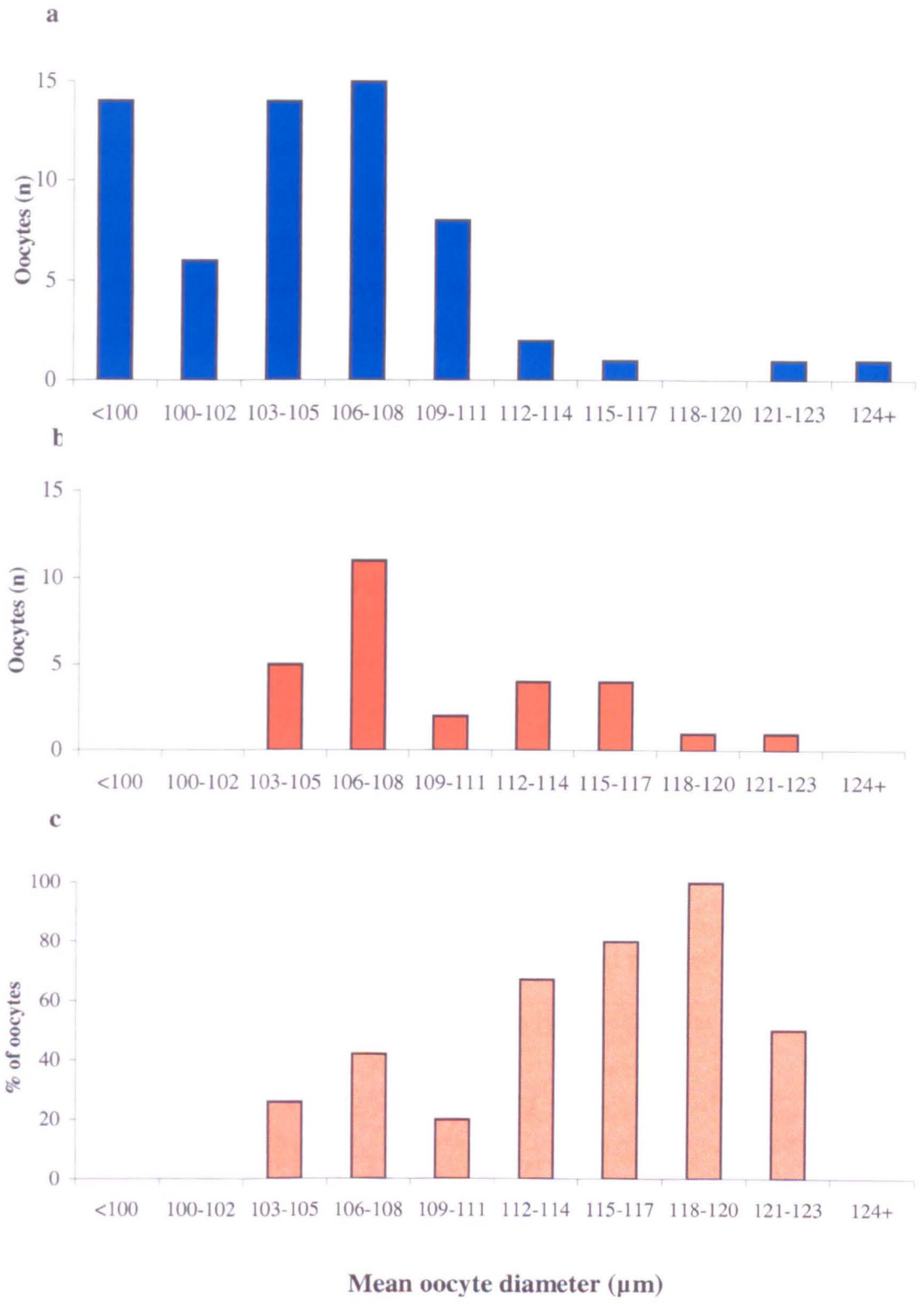


Figure 7.2 Frequency histogram showing oocyte diameters for patients having unstimulated polycystic ovaries on day 0 (day of ICSI for mature oocytes)
a) Immature or atretic oocytes
b) Mature oocytes
c) Oocytes that progressed to maturity expressed as a percentage of viable oocytes on day 0.

Table 7.3 shows the effects of the levels of cumulus cover on oocytes at collection (day-2) recovered from patients with PCO on outcome of in vitro culture. 49% of oocytes retrieved from unstimulated ovaries had some cumulus cells attached and this was associated with a tendency towards improved maturation. Table 7.4 shows mean diameters of oocytes from patients with PCO in relation to the outcome of in vitro culture according to cumulus grade at collection (day-2). The diameters of oocytes with dense cumulus cover at collection were not significantly different to those with less or no cumulus cover, so although ~33% of oocytes could not be measured on day-2 due to a dense cumulus cover (see table 7.2) those that could (14/128, 11%) did not differ significantly from those with less or no cumulus cover (Table 7.4).

Table 7.3 Effects of the levels of cumulus cover on oocytes at collection (day-2) recovered from patients with PCO on outcome of in vitro culture.

Cumulus grade	Mature	Immature	Atretic
0	13	23	29
1	10	6	1
2	2	3	0
3	13	20	8

Table 7.4 Mean diameters of oocytes from patients with PCO in relation to the outcome of in vitro culture according to cumulus grade at collection (day-2)

		Mean oocyte diameter (μm)		
Cumulus grade	**Outcome**	**Day-2**	**Day-1**	**0**
0	Mature	**107** ± 3.9	**107** ± 5.6	**108** ± 4.1
	Immature/atretic	**106** ± 5.5	**106** ± 5.8	**104** ± 8.4
1	Mature	**109** ± 3.7	**110** ± 3.6	**110** ± 5.3
	Immature/atretic	**107** ± 2.3	**108** ± 0.4	**107** ± 2.2
2	Mature		**110**	**104**
	Immature/atretic	**97**	**106** ± 6.0	**106** ± 2.5
3	Mature	**106** ± 2.7	**108** ± 4.9	**112** ± 4.4
	Immature/atretic	**103** ± 4.5	**106** ± 4.5	**107** ± 12.0

Mean ± SD

Key for Tables 7.3 and 7.4: 0 = devoid of any cumulus/ no more than 10 scattered cells; 1 = partial cover; 2 = complete cover; 3 = substantial multilayered cover.

Figure 7.3 shows the relationship between the diameters of in vitro matured oocytes from patients with PCO on day 0 and their ability to fertilize and cleave when injected with sperm from a fertile donor. The minimum diameter of a mature oocyte in this group on day 0 was 103μm, although only two oocytes were of this size. The median oocyte diameter of mature oocytes on day 0 was 108μm, interquartile ranges = 106-113, n = 28 and the range = 103-121μm.

The minimum diameter of an oocyte on day 0 which fertilized was 105μm and the median oocyte diameter on day 0 for oocytes that subsequently fertilized was 112.5μm, interquartile ranges = 107.25-116, n = 10 and the range = 105-121μm. Of the mature oocytes on day 0, only 18% in the smallest diameter group (103-106μm) subsequently fertilized after ICSI (Figure 7.3b). Between 103 and 118μm, there was an increasing trend in fertilization with increasing diameter, although the numbers of oocytes available at larger diameters were reducing, making statistical analysis unreliable.

Figure 7.3c shows the relationship between the mature oocyte diameter on day 0 and the ability of fertilized oocytes to cleave. The smallest oocyte to cleave, following fertilization had a diameter of 105μm on day 0, however the median oocyte diameter on day 0 was 112.5μm, interquartile ranges = 107.75-116.25, n = 8 and the range = 105-121μm. 8/10 oocytes that fertilized subsequently cleaved, although low numbers of measurements preclude any further analysis.

These data suggest the size threshold for oocyte maturation to be a major limiting factor. The majority of oocytes that mature in vitro appear not to be limited by their size from subsequent development, at least to fertilization and cleavage, although a tendency towards increased maturation and fertilization with increasing oocyte diameter was apparent.

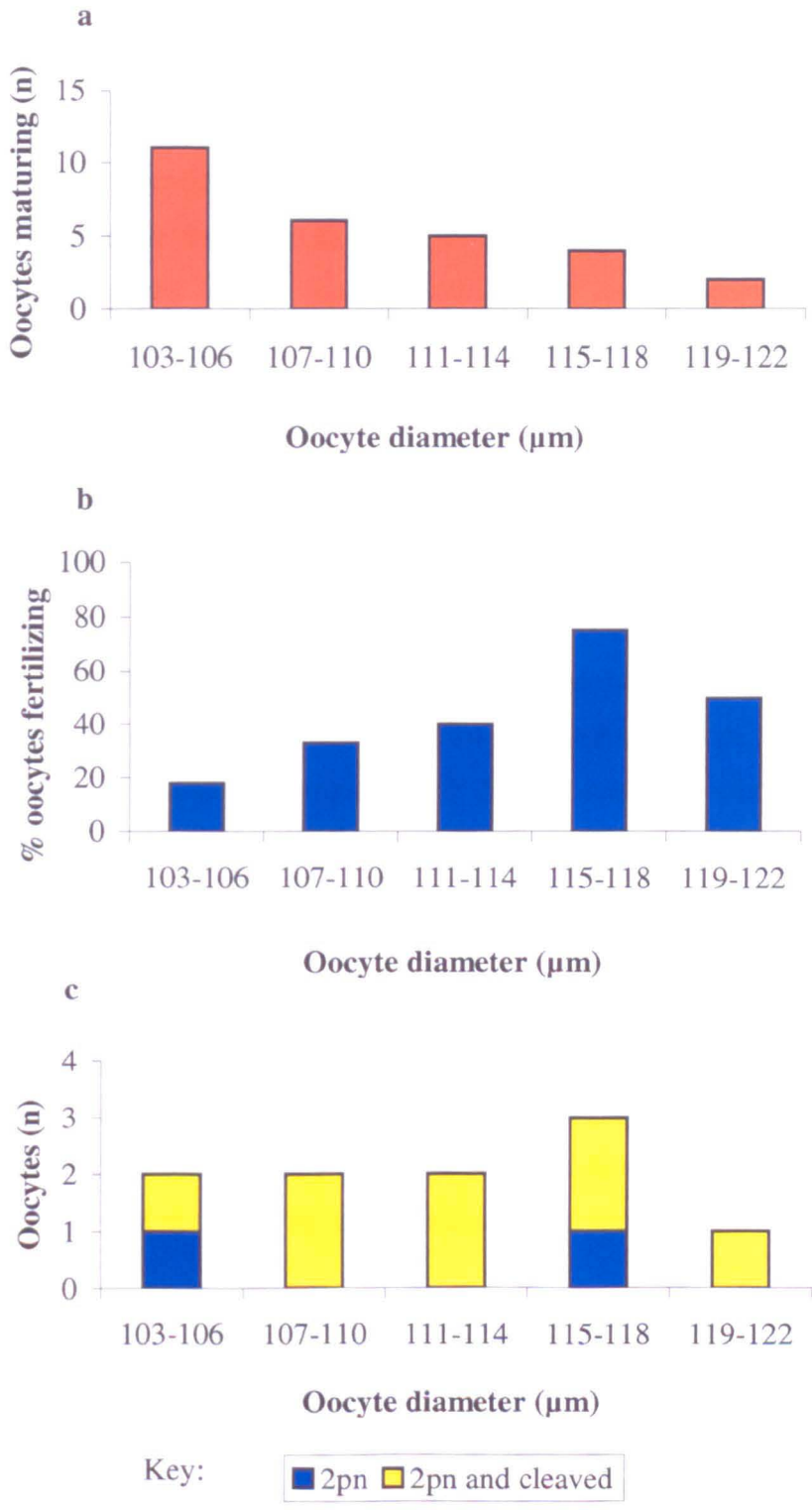


Figure 7.3 Diameters of in vitro matured oocytes on day 0 obtained from unstimulated patients with PCO in relation to their competence to fertilize and cleave.

a) Frequency histogram of diameters of in vitro matured oocytes on day 0.

b) Proportion of mature oocytes fertilizing (2pn) according to their diameter on day 0.

c) Frequency histogram of fertilized oocytes undergoing cleavage or arresting at the 2pn stage according to the oocyte diameter on day 0.

Figure 7.4 shows the relationship between time and oocyte diameter during in vitro culture with and without FF-MAS. The diameter measurements obtained from individual oocytes were plotted between days–2 and 0 according to the outcome of culture and the conditions to which they were exposed (0, 10, 30 μg/ml FF-MAS). Similar graphs were obtained using full measurement sets only (i.e. three measurements, day-2, -1 and 0 for each oocyte) or if incomplete data sets were included.

Different growth patterns in the mean diameters of oocytes were apparent according to whether they became mature, remained immature or underwent atresia. A similar pattern was observed in all culture conditions (Figure 7.4). The oocytes that matured in each culture condition were the largest oocytes by day 0 and the oocytes that became atretic in all culture conditions decreased in size from day-2 to day 0 to become the smallest oocytes on day 0. The reducing diameters of oocytes that became atretic in culture was significantly different between day-2 and day 0 for those cultured in 10μg/ml FF-MAS only ($p < 0.05$), although a similar trend was evident in all conditions.

In both FF-MAS culture conditions (30 and 10μg/ml) the mean oocyte diameters of mature, immature and atretic oocytes on day 0 (Figure 7.4b and c) were significantly different ($p < 0.05$) despite their diameters on day-2 being similar. Interestingly, this difference did not occur in oocytes cultured in control conditions (Figure 7.4a).

Oocyte growth during culture (from day-2 to day 0) for those oocytes that matured was tested for statistical significance using the non-parametric sign test according to the conditions of culture to which oocytes were exposed, revealing that the extent of growth over the two-day period was not significantly different among the three culture conditions. The difference in growth between oocytes maturing in 30μg/ml FF-MAS and in control conditions was also assessed using a two-sample t-test. No significant difference was found, although the t-value of 1.806 lies between the 5-10% probability range, indicating a trend towards increased growth between day-2 and day 0 in 30μg/ml FF-MAS compared to control.

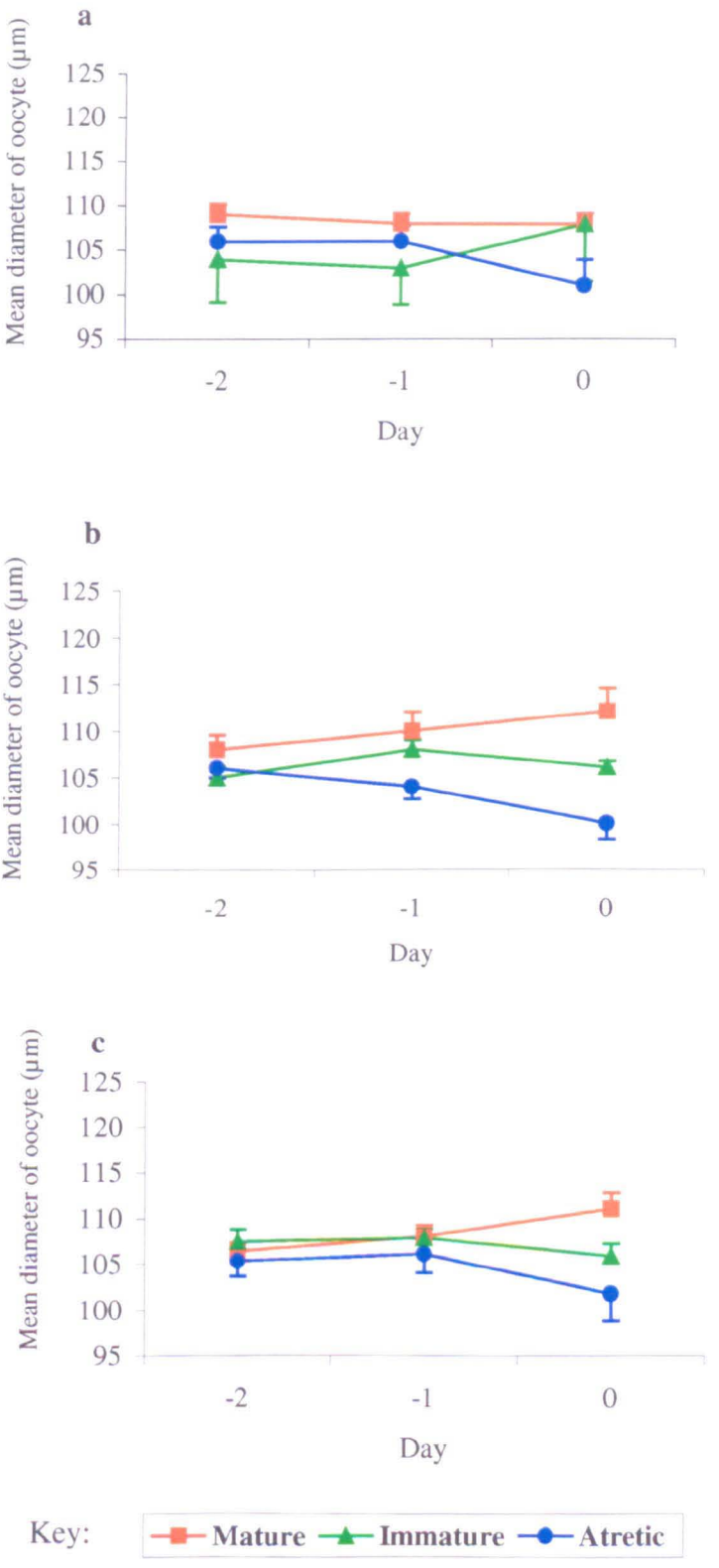


Figure 7.4 Relationship between time and oocyte diameter during in vitro culture in a) control, b) 10µg/ml FF-MAS and c) 30µg/ml FF-MAS. These oocytes were collected on day-2 from unstimulated patients with PCO. Results are expressed as mean ± SEM.

7.2.2 Oocyte + zona diameter

The majority (79%) of measurements of viable oocytes had mean diameters (including zona) in the 146-163μm range on day-2 (Figure 7.5a). Figure 7.5b shows the measurements including zona, on day-2 of oocytes that subsequently matured in vitro, 70% of these were in the 152-163μm range.

The most common size range for immature/atretic oocytes on day 0 was 152-157μm with 34% of oocyte diameters (including zona) falling in the size range (Figure 7.6a). One abnormally large oocyte measuring 192μm on day 0 was probably diploid. The majority of oocytes + zonas that matured on day 0 were larger than those remaining immature or undergoing atresia, 46% of oocytes + zonas had diameters in the 158-163μm size range, and 62% of mature oocytes had diameters over 158μm on day 0 (Figure 7.6b). Figure 7.7 shows the relationship between the oocyte (including zona) diameter and the ability to fertilize and cleave. The smallest oocyte (including zona) which matured had a diameter on d 0 of 145μm, 46% of oocytes had diameters in the 158-163μm size range, and 62% were over 158μm (Figure 7.6b). Of the oocytes in the 158-163μm size range 50% fertilized. The one oocyte with a diameter of 170μm that matured subsequently fertilized and cleaved. Low numbers of measurements of this type preclude any further analysis.

There was no significant difference between the mean diameter of oocyte + zona between day-2 and day 0 within each culture group. The oocyte + zona data on day-2 and day 0 did not reveal significant differences between oocytes (including zona) measuring 140-151μm and those measuring 152-175μm and the ability of the oocyte to mature in vitro. However, on day 0, there was a significant difference ($p < 0.05$) between oocytes (including zona) measuring 140-157μm and those measuring 158-175μm in the oocyte's maturity. This significant difference was not due to the presence or absence of FF MAS during culture (Figure 7.8).

Therefore, while there exist several similarities in the results of oocyte diameter and oocyte including zona measurements, the dimensions of the oocyte itself appear to have a greater discriminatory ability than the combined oocyte including zona measurement.

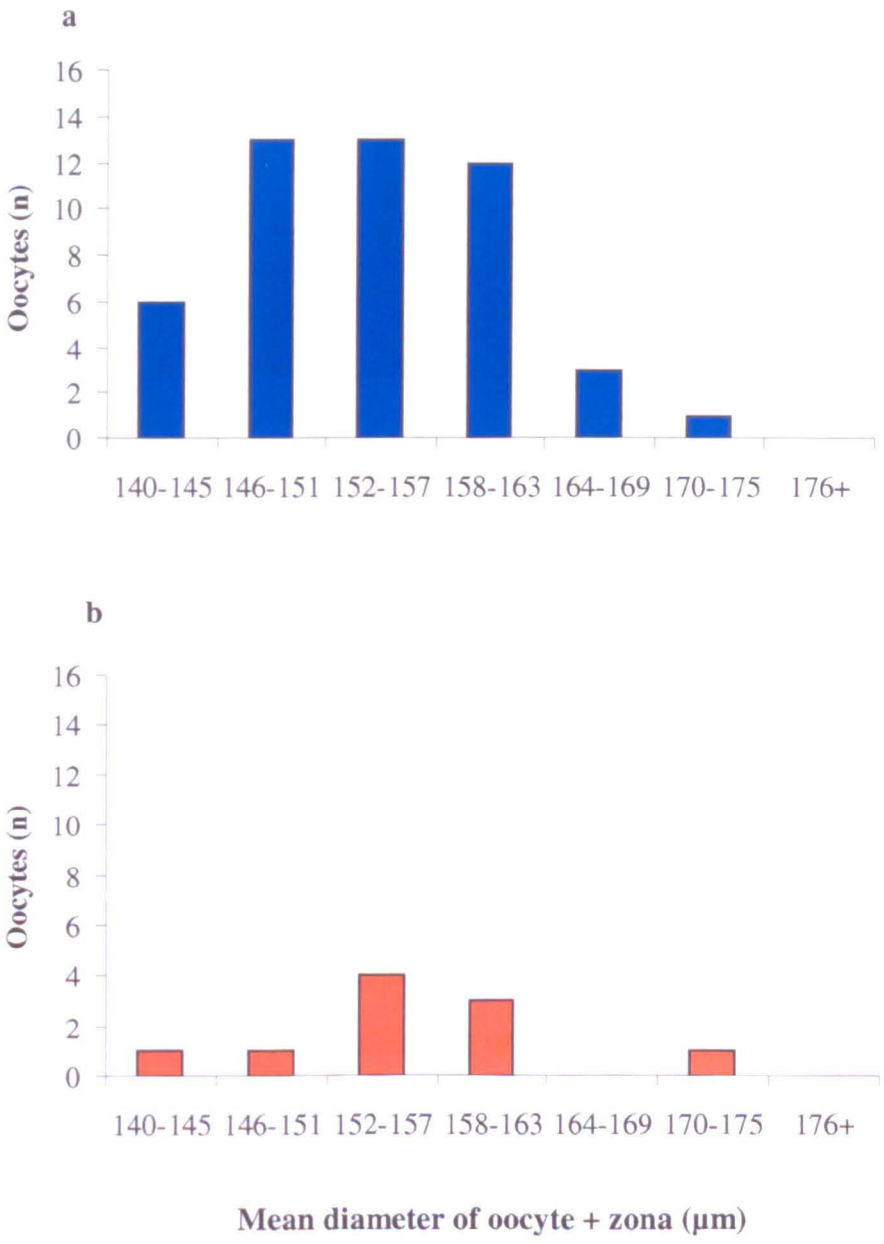


Figure 7.5 Frequency histogram showing mean oocyte diameters (including zona) on day of collection (day-2) of oocytes from unstimulated patients having PCO
a) Viable oocytes
b) Oocytes becoming mature in vitro

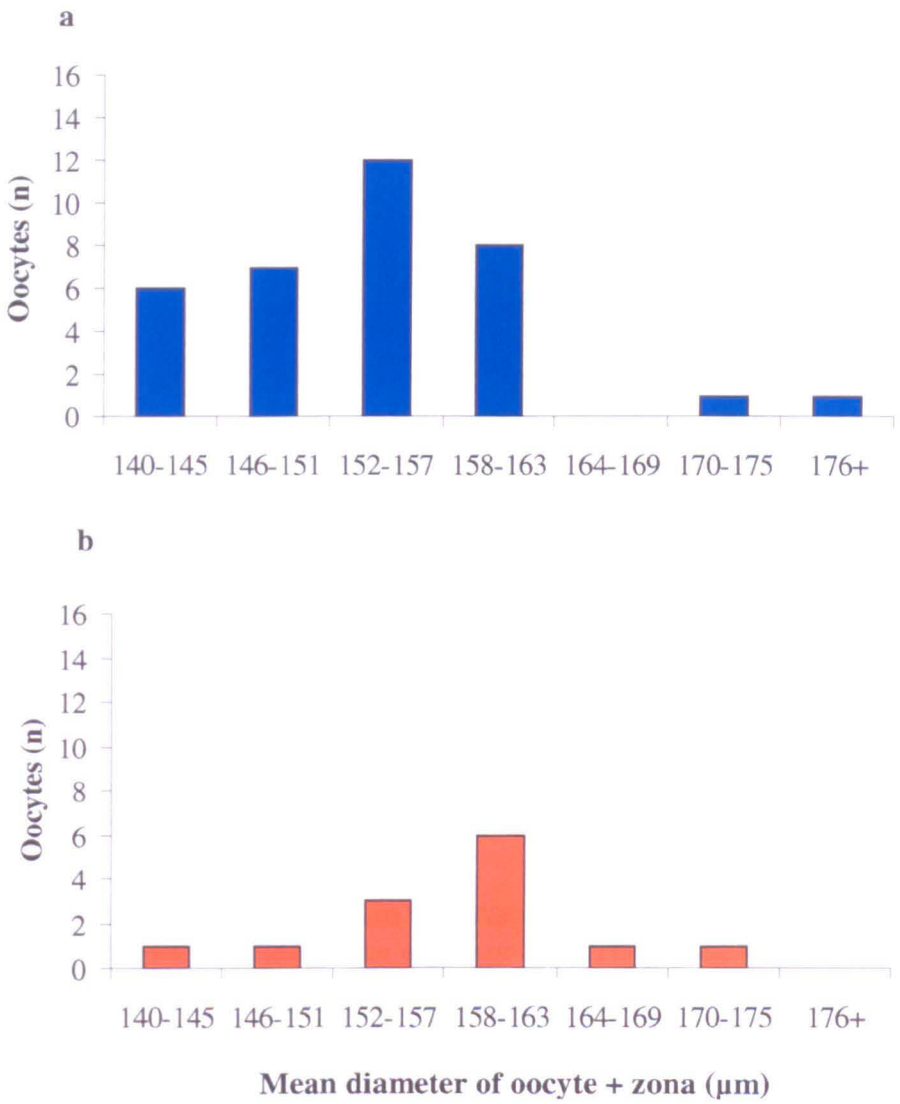


Figure 7.6 Frequency histogram showing mean oocyte diameters (including zona) on day of ICSI (day 0) of oocytes from unstimulated patients having PCO.
a) Immature or atretic oocytes
b) Mature oocytes

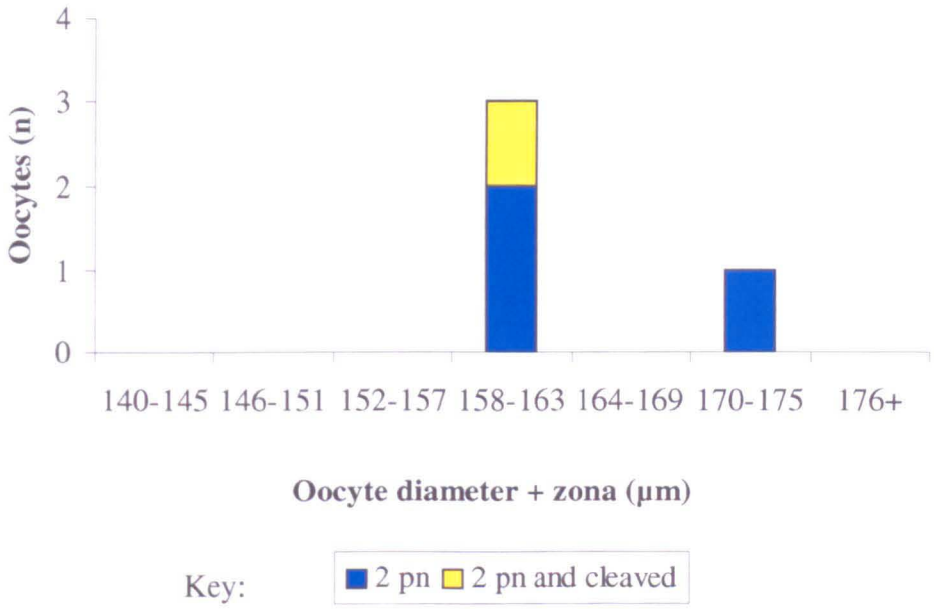


Figure 7.7 Frequency histogram of fertilized oocytes obtained from unstimulated patients with PCO, undergoing cleavage or arresting at the 2pn stage according to the oocyte + zona diameter on day 0.

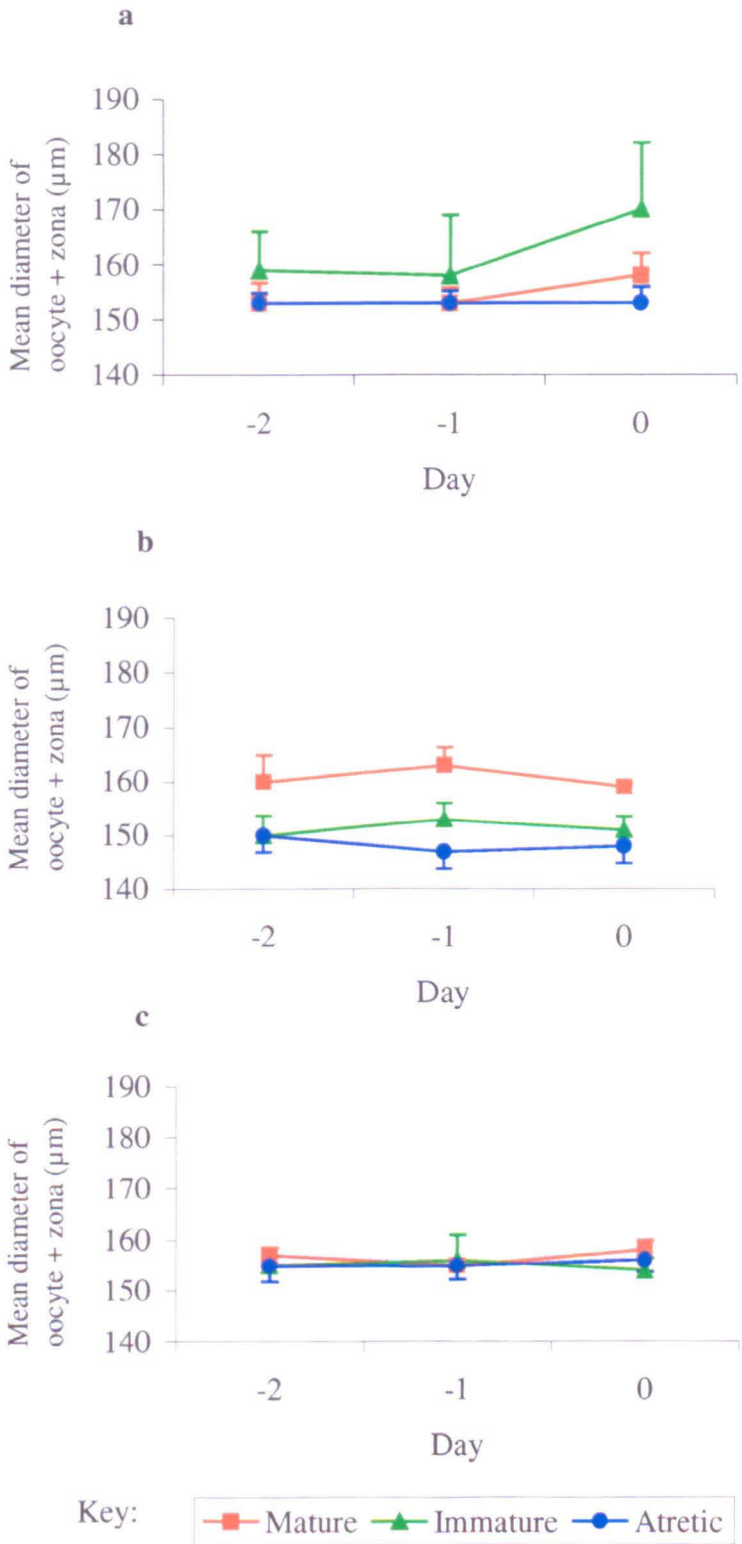


Figure 7.8 Relationship between time and oocyte + zona diameter during in vitro culture in a) control, b) 10µg/ml FF-MAS and c) 30µg/ml FF-MAS. These oocytes were collected on day-2 from unstimulated patients with PCO. Results are expressed as mean ± SEM.

7.2.3 Zona pellucida thickness

Figure 7.9a presents the frequency distribution of the mean zona pellucida thickness of viable oocytes collected from unstimulated patients with PCO on day-2. This distribution was approximately normal. The most common size range was 21-22μm (34%) and the majority (77%; 34/44) of oocytes collected had a mean zona pellucida thickness of between 19-24μm on the day of collection.

The measurements of zona pellucida thickness on day-2 of oocytes that subsequently matured in vitro showed that 60% measured between 21-22μm (Figure 7.9b) although numbers were low.

Figure 7.10 shows the measurements on day 0 of mean zona pellucida thickness of oocytes that failed to mature or underwent atresia compared to those, which matured. There was no significant difference in the distribution of zona pellucida thickness between these two groups. Interestingly, the broad distribution on day 0 was noticeably different to that observed at collection on day-2 (compare with Figure 7.9) suggesting some modification in thickness of the zona pellucida in culture.

Figure 7.11 shows the relationship between time and zona pellucida thickness during in vitro culture with and without FF-MAS. Changes in zona pellucida thickness were not related to the presence or absence of FF-MAS.

Table 7.5 shows the zona pellucida thickness on day +1 (day after insemination) of oocytes that fertilized and those that failed to fertilize or became atretic and on day+2 of embryos or oocytes that failed to fertilize or became atretic after ICSI insemination on day+1. There was little change in zona pellucida thickness between day +1 and day+2 in either group, however, the fertilized oocytes tended towards significantly thicker zonas ($p<0.05$).

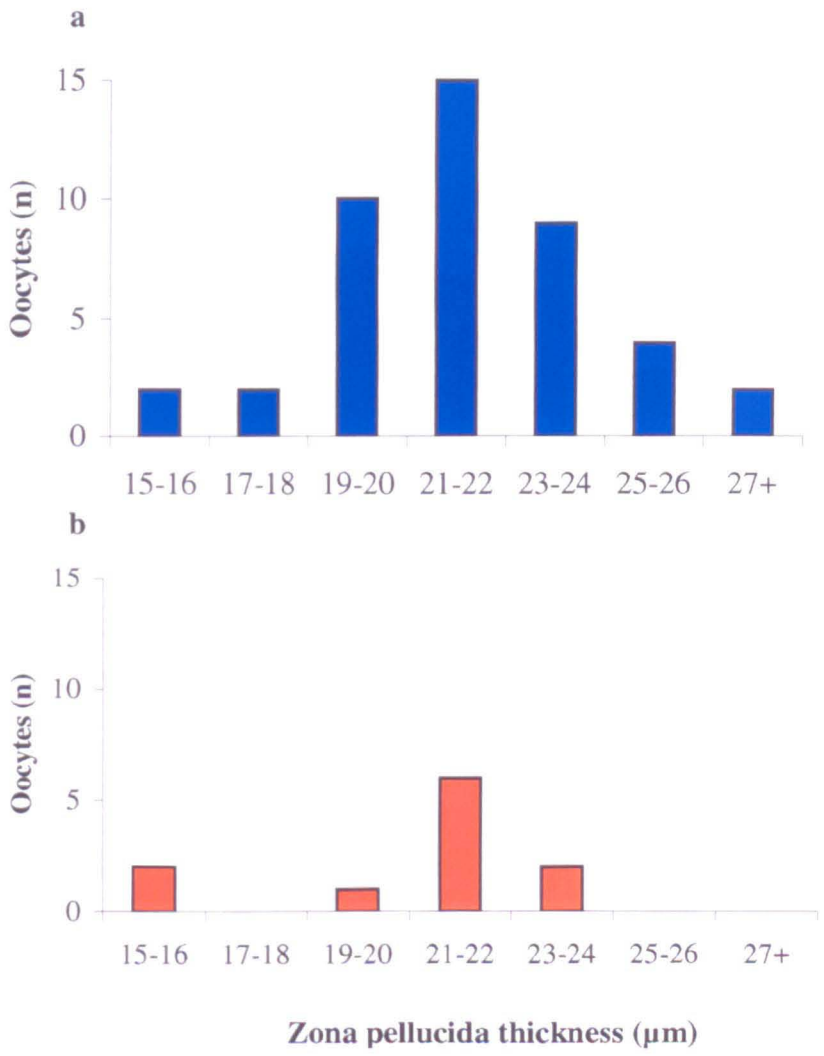


Figure 7.9 Frequency histogram showing mean zona pellucida thickness on day of collection (day-2) of oocytes from unstimulated patients having PCO
a) Viable oocytes
b) Oocytes becoming mature in vitro

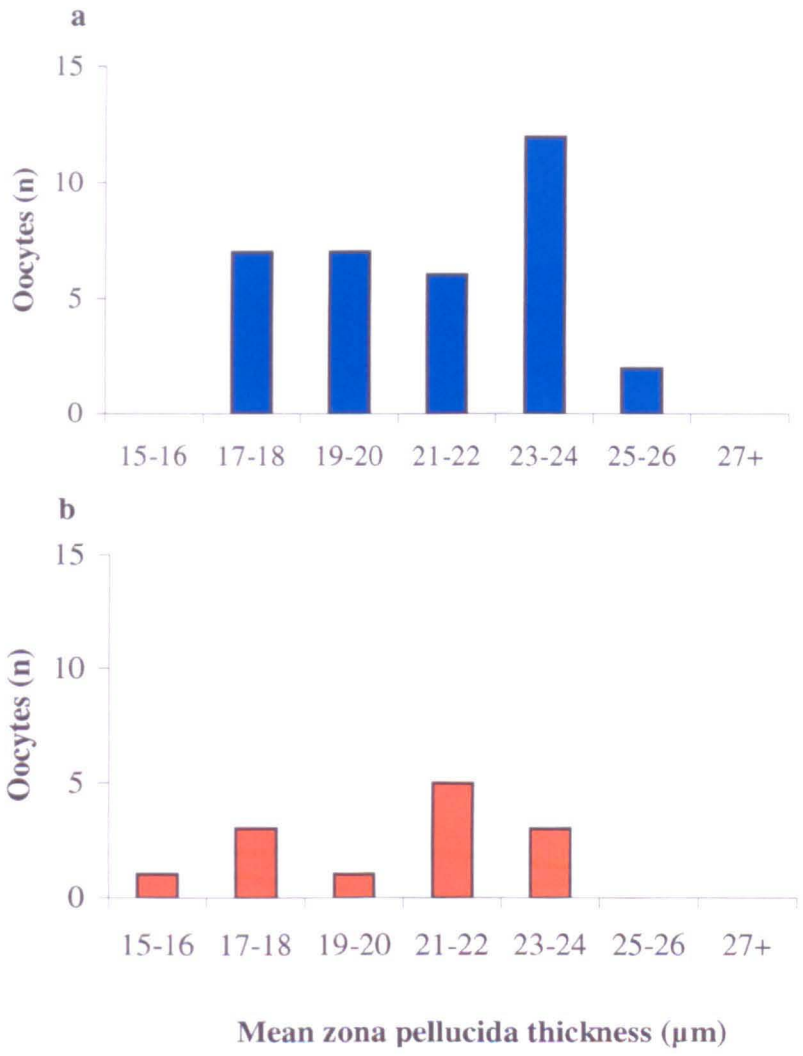


Figure 7.10 Frequency histogram showing mean zona pellucida thickness on day 0 (day of ICSI for mature oocytes) of oocytes from unstimulated patients having PCO
a) Immature or atretic oocytes
b) Mature oocytes

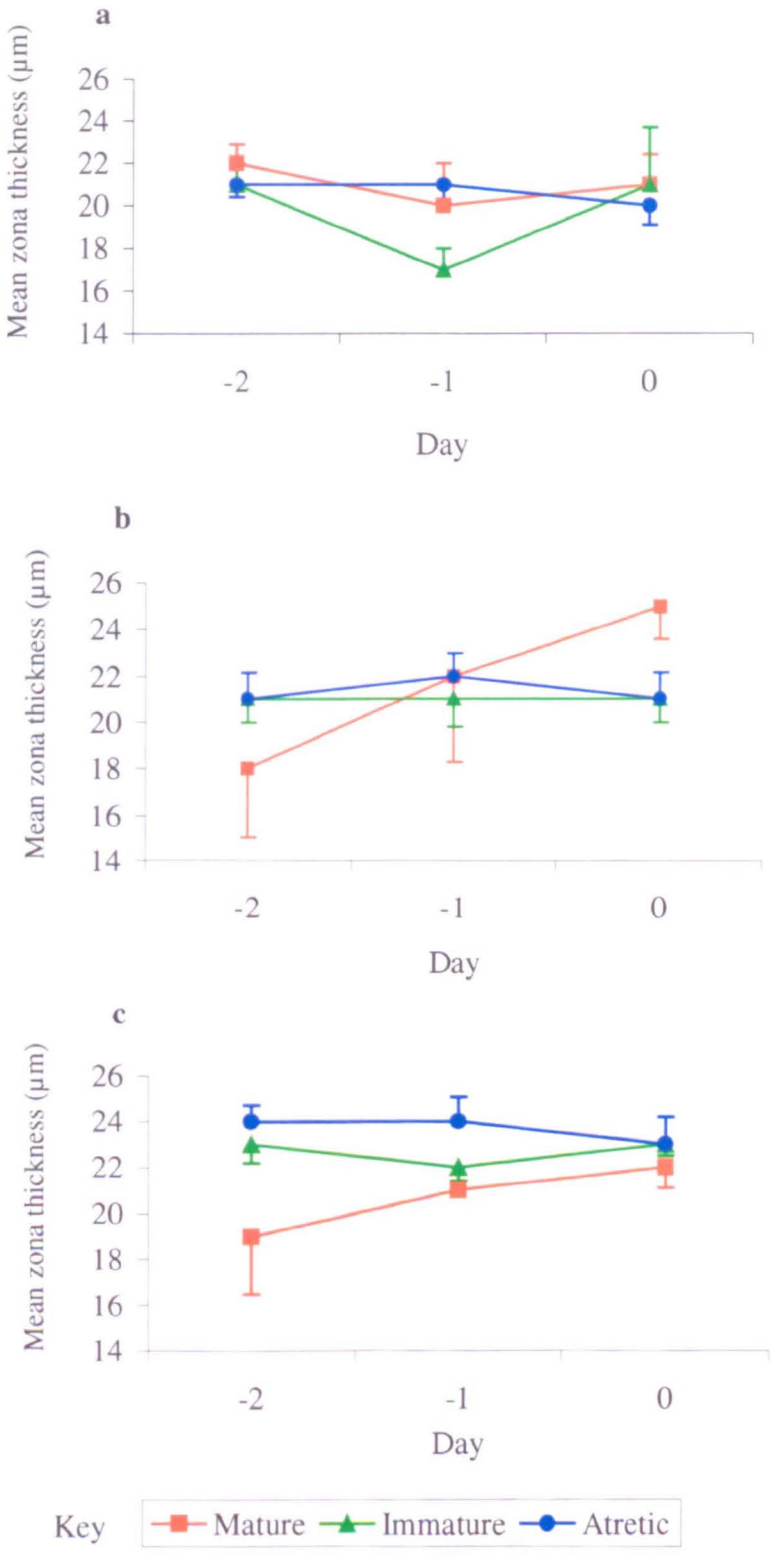


Figure 7.11 Relationship between time and zona pellucida thickness during in vitro culture in a) control, b) 10µg/ml FF-MAS and c) 30µg/ml FF-MAS. These oocytes were collected on day-2 from unstimulated patients with PCO. Results are expressed as mean ± SEM.

Table 7.5 Zona pellucida thickness on day+1 and day+2 of oocytes collected from patients with PCO that fertilized after ICSI insemination on day+1 and those that failed to fertilize or became atretic

	Mean zona pellucida thickness (μm)	
	Day+1	**Day+2**
Fertilized	21.8 ± 1.9	20.1 ± 1.9
Range	19.8-24.2	15.5-24.2
Unfertilized/atretic	16.9 ± 2.7	17.2 ± 3.4
Range	11.6-21.5	12.5-22.0

Mean ± SD

7.2.4 Perivitelline space (PVS)

Figure 7.12a presents the frequency histogram of the mean PVS of viable oocytes collected from unstimulated patients with PCO on the day of collection (day-2). The most common size range was 3-4μm. The measurements of mean PVS of oocytes that became mature in vitro were spread out between 1-6μm (Figure 7.12b).

Figure 7.13 shows the frequency histogram of the mean PVS of oocytes from unstimulated patients with PCO on day 0 (the day of ICSI of mature oocytes) according to whether the oocytes matured in vitro. 50% of oocytes that remained immature or became atretic had a mean PVS of 3-4μm; there was a broad distribution of PVS in oocytes that matured in vitro (Figure7.13b).

Figures 7.14 shows the relationship between time and mean PVS during in vitro culture with and without FF-MAS. The data do not indicate any consistent relationship between PVS and oocyte maturation in vitro, furthermore FF-MAS does not appear to relate to this measurement. There were no significant differences between the groups.

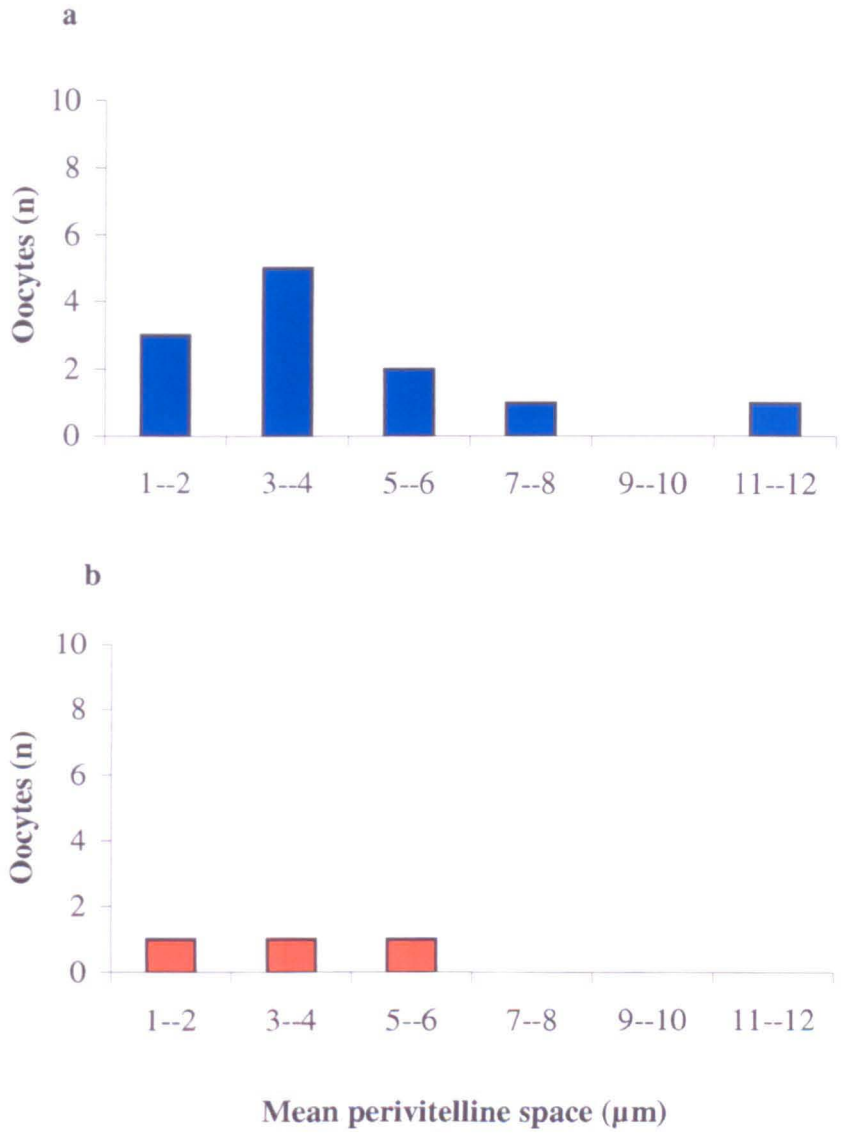


Figure 7.12 Frequency histogram showing mean perivitelline space on day of collection (day-2) of oocytes from unstimulated patients with PCO.
a) Viable oocytes
b) Oocytes becoming mature in vitro

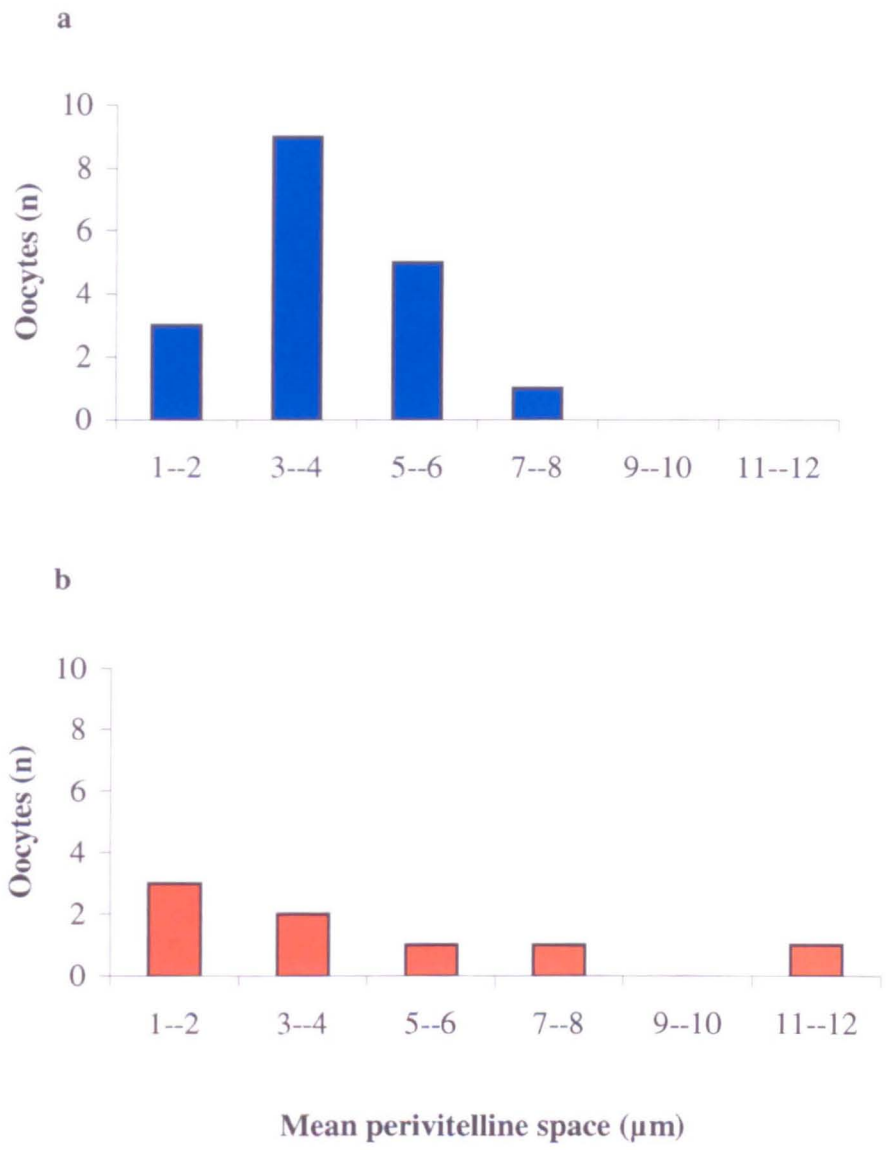


Figure 7.13 Frequency histogram showing mean perivitelline space on day 0 (day of ICSI for mature oocytes) of oocytes from unstimulated patients with PCO.
a) Immature or atretic oocytes
b) Mature oocytes

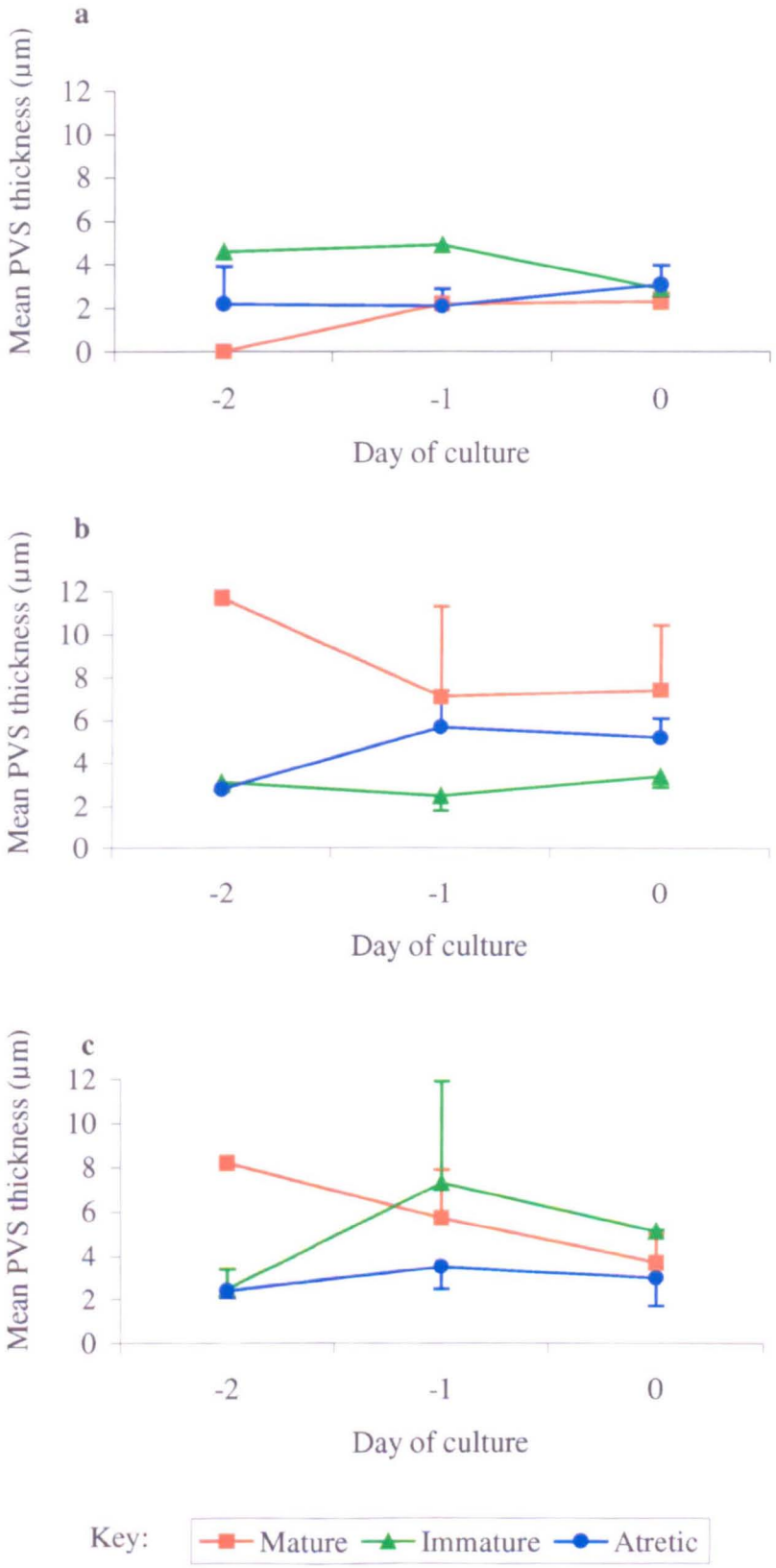


Figure 7.14 Relationship between time and mean perivitelline space (PVS) during in vitro culture in a) control, b) 10µg/ml FF-MAS and c) 30µg/ml FF-MAS collected on day-2 from patients with PCO.
Results are expressed as mean ± SEM.

7.3 Oocytes from patients undergoing ICSI treatment, cultured with or without MAS

7.3.1 Oocyte diameter

Figure 7.15a presents the distribution of the mean diameter of viable immature oocytes donated by patients undergoing ICSI treatment on the day of oocyte recovery (day-2). The distribution is approximately normal; the most common size range was 109-111μm (36% of oocytes). The measurements of those that went on to mature in vitro did not follow a normal distribution (Figure 7.15b); all except one had mean diameters of >106μm on the day of collection (day-2).

In this study, the smallest oocyte that matured had an initial diameter of 99μm on day-2, whereas the majority of oocytes maturing had diameters of at least 106μm. Interestingly, none of the seven oocytes with initial diameters of ≥115μm matured.

Comparison of Figure 7.15 with 7.1 shows that the oocytes donated by ICSI patients were significantly larger at the time of collection than those from PCO ovaries ($p<0.001$).

Figure 7.16 shows the diameters of oocytes on day 0 according to whether they matured in vitro. Comparison with Figure 7.15 shows that many oocytes had grown in vitro during the culture period resulting in a modal diameter of 112-114μm in both mature oocytes and those that remained immature. There was no significant difference in size between the two groups of mature and immature/atretic oocytes (Figure 7.16).

There was no apparent relationship between mean oocyte diameter and the likelihood of maturation in these oocytes derived from ICSI patients in contrast to those derived from patients with PCO (Figure 7.2). The small number of oocytes of a small size precludes any comment on a threshold size for maturation in oocytes from this source.

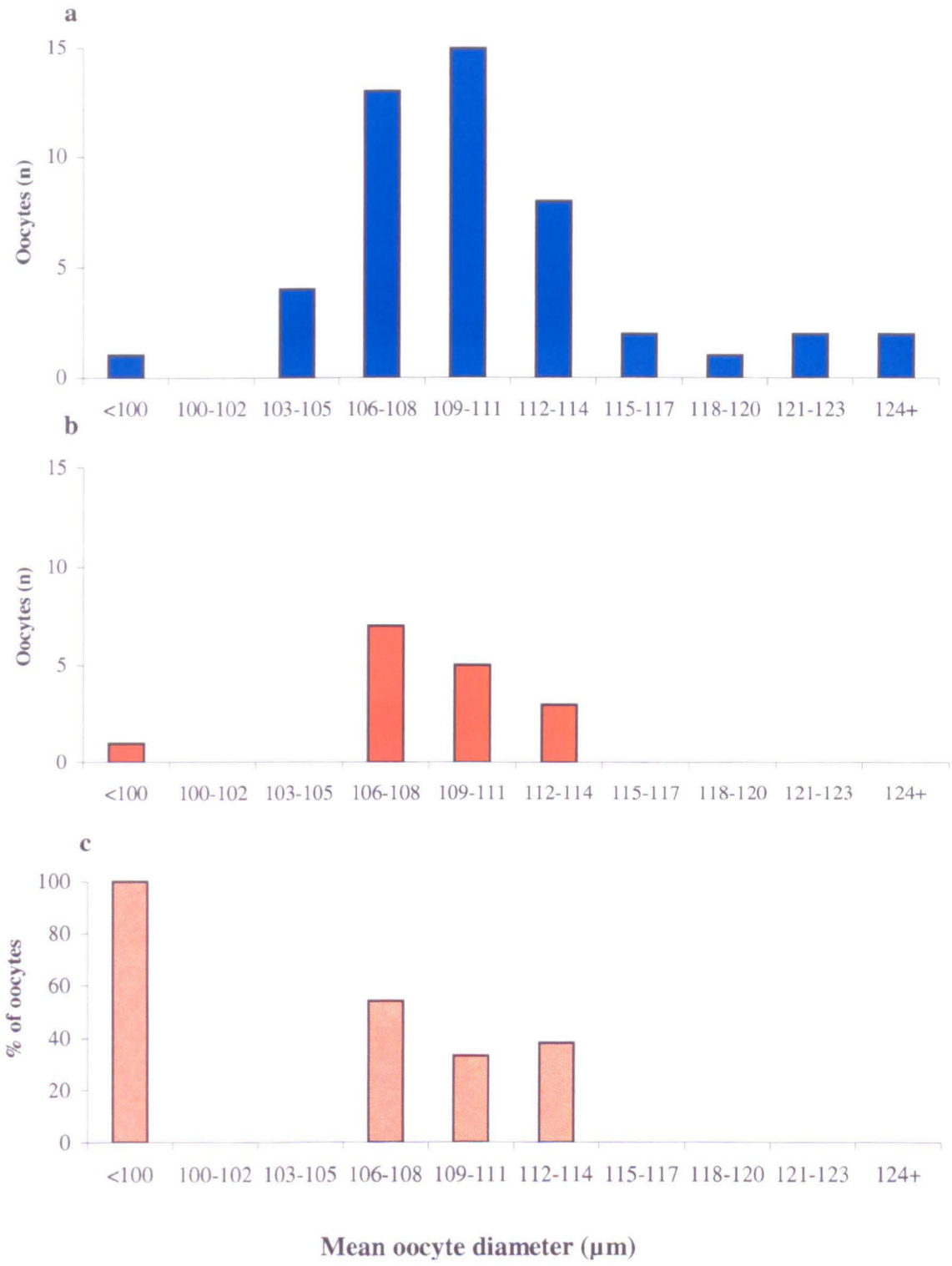


Figure 7.15 Frequency histogram showing mean oocyte diameters on day of collection of immature oocytes from patients undergoing ICSI treatment.
a) Viable oocytes
b) Oocytes becoming mature in vitro
c) Oocytes that progressed to maturity expressed as a percentage of viable oocytes on day of collection.

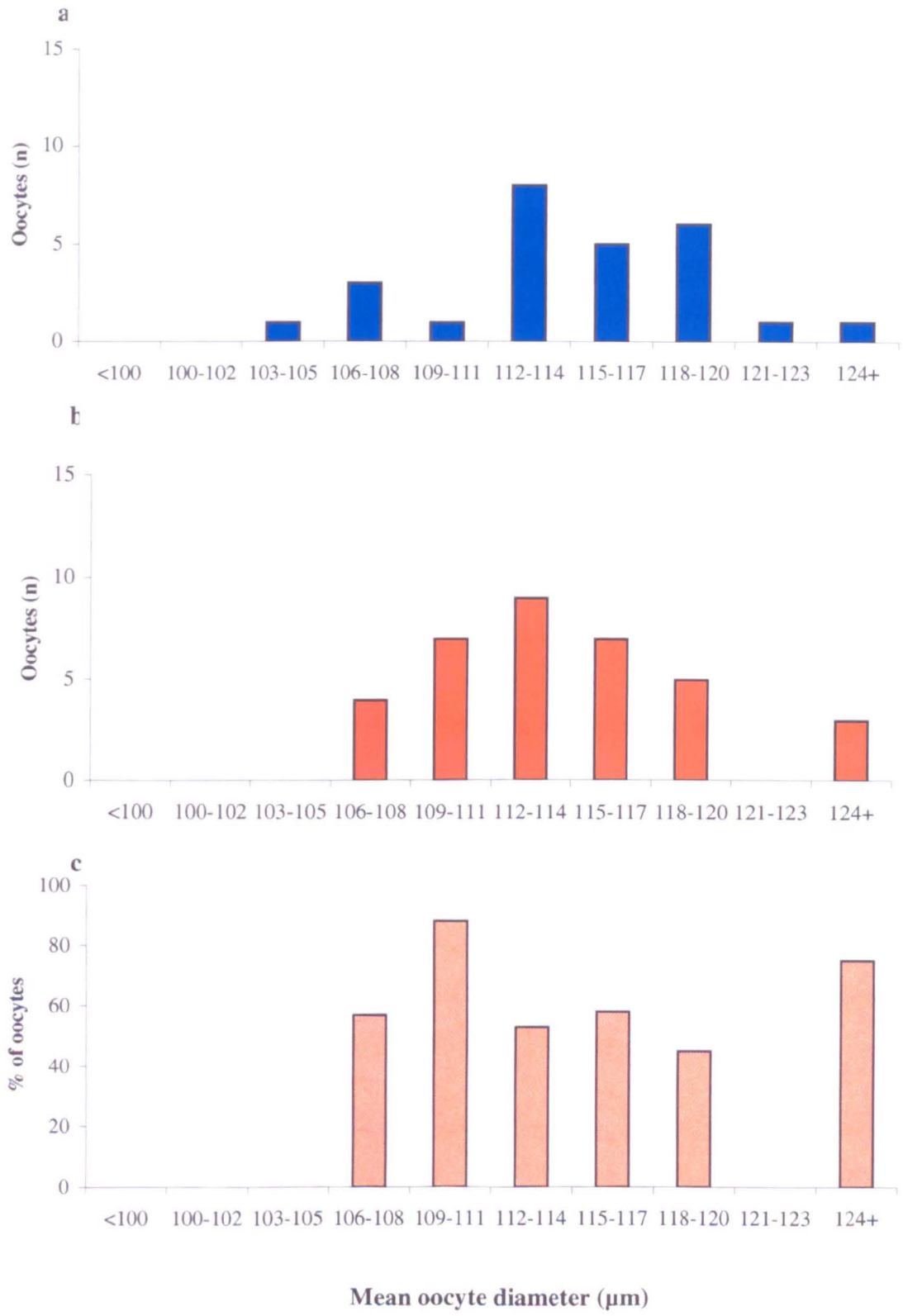


Figure 7.16 Frequency histogram showing mean oocyte diameters for patients undergoing ICSI treatment on day 0 (day of ICSI for mature oocytes)
a) Immature or atretic oocytes
b) Mature oocytes
c) Oocytes that progressed to maturity expressed as a percentage of viable oocytes on day 0.

Figure 7.17 shows the relationship between the diameters of in vitro matured oocytes on day 0 and their subsequent fertilization and cleavage. The minimum oocyte diameter associated with maturation was 106μm on day 0, compared with 99μm on day of oocyte collection. Very few oocytes had smaller diameters, so a threshold could not be attributed. The median oocyte diameter for mature oocytes on day 0 was 114μm, interquartile ranges = 110-116, n = 35 and the range = 106-131μm.
Figure 7.17c shows all the oocytes that fertilized or both fertilized and cleaved, whereas Figure 7.18a and b presents oocytes maturing in 23-24 hr and 46-48 hr respectively. The smallest oocyte fertilizing after ICSI had a diameter of 106μm on day 0, the median oocyte diameter on day 0 for oocytes that were competent to fertilize was 113μm, interquartile ranges = 109-114, n = 13 and the range = 106-125μm. For competence to cleave after fertilization, the minimum oocyte diameter on day 0 was 107μm, however the median oocyte diameter on day 0 was 113μm, interquartile ranges = 111-117, n = 7 and the range = 107-125μm.

Figure 7.18 shows the numbers of oocytes fertilizing and cleaving after maturation in vitro according to oocyte diameter. Figure 7.18a shows, of oocytes that matured within 23-24 hr of in vitro culture, 2/6 (33%) of the fertilized oocytes subsequently cleaved. However, of oocytes that matured within 46-48 hr of in vitro culture, 5/7 (71%) fertilized oocytes subsequently cleaved (Figure 7.18b). While this may provide some suggestion that a prolonged period of maturation could be associated with improved cleavage potential, the numbers of embryos cleaving, and the subsequent measurements available were too few for meaningful analysis.

Among the ICSI patients, 50% of oocytes maturing in vitro had matured by 23-24 h (Chapter 5, Figure 5.6). For oocytes maturing in 23-24 hr, the median oocyte diameter was 113μm, interquartile ranges = 110-113.75 and for oocytes maturing in 46-48 hr, the median oocyte diameter was 112μm, interquartile ranges = 108.5-114.5. There was no significant difference in oocyte diameter on day 0 between oocytes maturing in 23-24 hr and those in 40-48 hr, within each culture group (control, 10μg/ml FF-MAS and 30μg/ml FF-MAS) or when pooling all the culture groups.

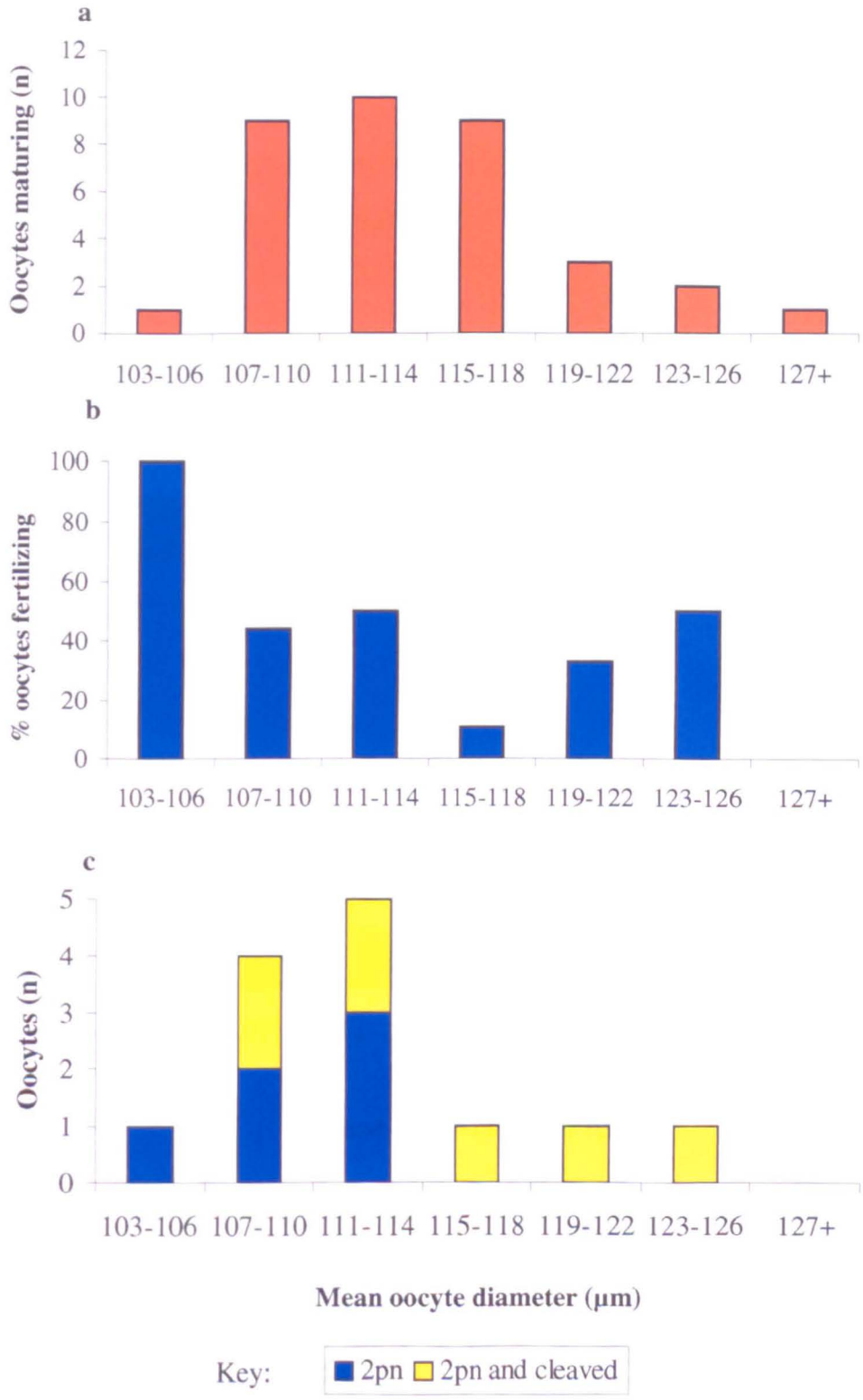


Figure 7.17 Diameters of in vitro matured oocytes on day 0 obtained from patients undergoing ICSI in relation to their competence to fertilize and cleave.
a) Frequency histogram of diameters of in vitro matured oocytes on day 0.
b) Proportion of mature oocytes fertilizing (2pn) according to their diameter on day 0.
c) Frequency histogram of fertilized oocytes undergoing cleavage or arresting at the 2pn stage according to the oocyte diameter on day 0.

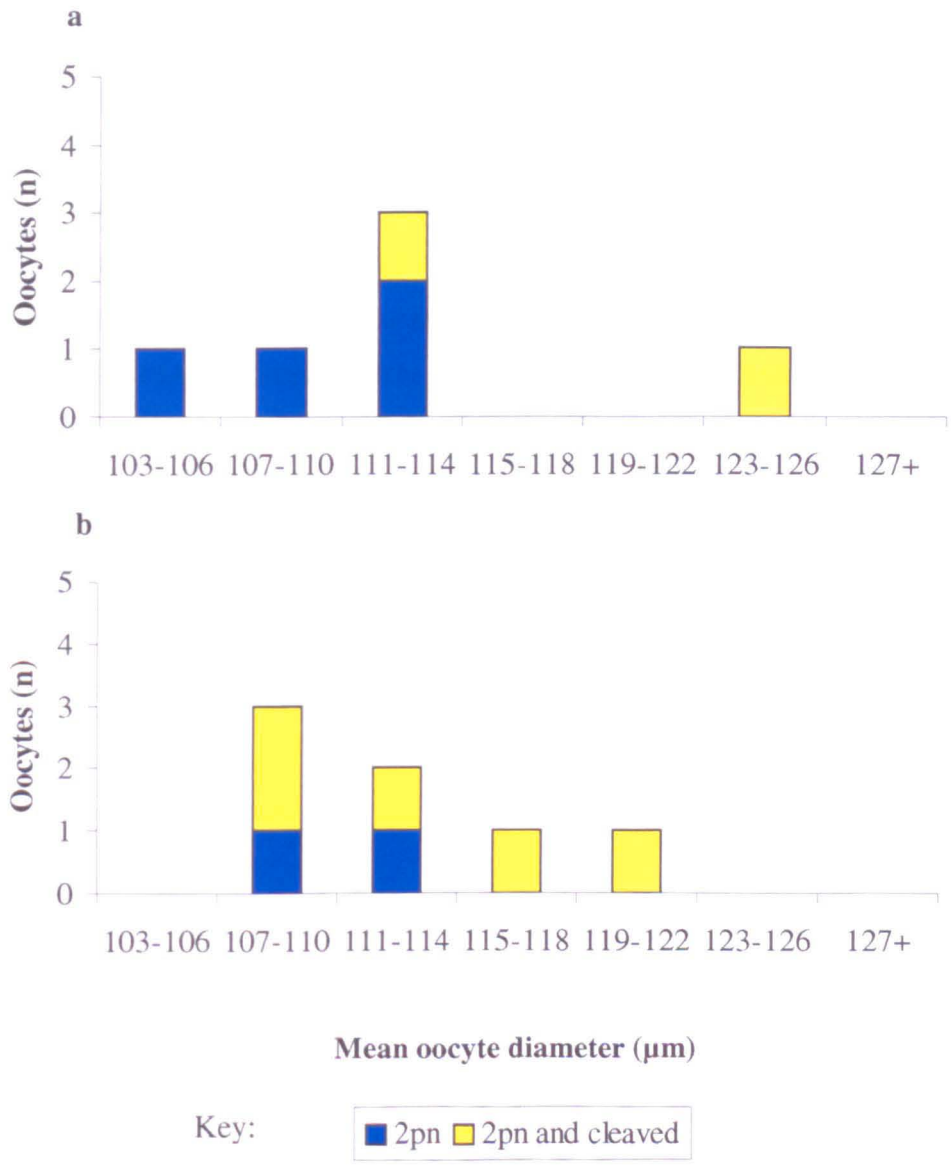


Figure 7.18 Numbers of oocytes donated by patients undergoing ICSI treatment, fertilizing and cleaving after maturation in vitro according to oocyte diameter
a) Oocytes that matured in 23-24 hr
b) Oocytes that matured in 46-48 hr

Figure 7.19 demonstrates the relationship between time and oocyte diameter during in vitro culture with and without FF-MAS. The growth patterns in Figure 7.19 were compared with those observed in oocytes retrieved from patients with PCO (Figure 7.4), revealing similarity in the patterns of mean oocyte diameter between the different subsets of oocytes (mature, immature and atretic) only for the 10μg/ml FF-MAS groups.

Oocyte diameter on the day of oocyte collection was compared to that on day 0 within each culture group and there were significant differences ($p<0.05$) in the diameters of oocytes on the day of collection and day 0 cultured in both 30μg/ml FF-MAS and 10μg/ml FF-MAS; however there was no significant difference for those oocytes cultured in control conditions. No significant difference was found within each culture group, when oocyte diameters on day 0 were compared in oocytes that matured and those that did not mature.

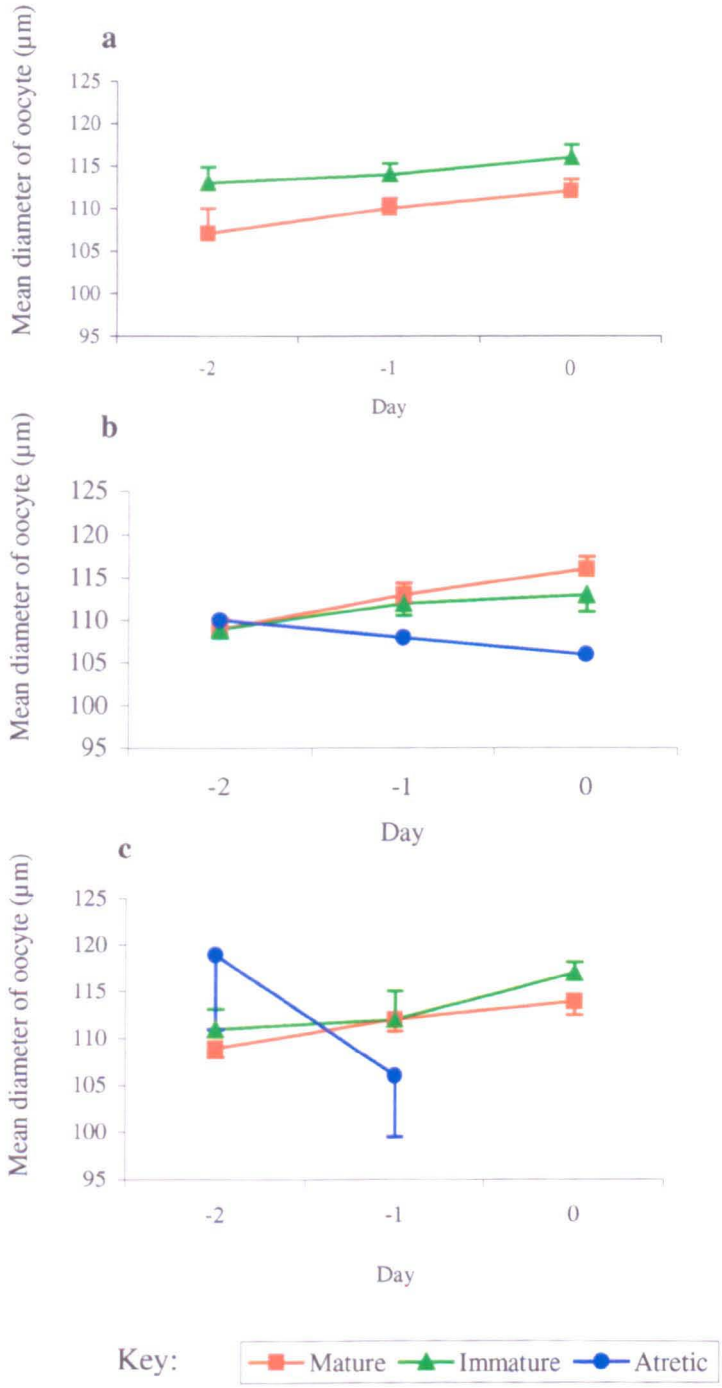


Figure 7.19 Relationship between time and oocyte diameter during in vitro culture in a) control, b) 10µg/ml FF-MAS and c) 30µg/ml FF-MAS. These oocytes were collected on day-2 from patients undergoing ICSI treatment. Results are expressed as mean ±SEM.

7.3.2 Oocyte + zona diameter

Figure 7.20 shows the frequency histogram of oocyte diameters on day of collection, giving an approximately normal distribution. The majority (80%) of viable oocytes had mean diameters (including zona) in the 152-169μm range on the day of collection (Figure 7.20a); the most common size range was 158-163μm (41%). Figure 7.20b shows the oocyte diameter (including zona) measurements on the day of collection of oocytes that subsequently matured in vitro, the majority being in the 152-169μm range.

Comparison of Figure 7.20 with Figure 7.5 shows that the oocytes (including zonas) donated by ICSI patients were significantly larger at the time of collection than those from polycystic ovaries ($p<0.001$).

Figure 7.21 shows the frequency histogram of oocyte diameters on day 0. The most common size range for immature and atretic oocytes on day 0 was 158-163μm with 41% of oocyte diameters (including zona) falling in this size range. The majority (52%) of oocytes that matured were in the same size category on day 0. There was no significant difference between the mean diameters of oocyte + zona between the day of collection and day 0 despite the extensive enlargement of oocyte cytoplasm that occurred over the same period (Figures 7.15 and 7.16).

The oocyte + zona data on the day of collection and day 0 did not reveal significant associations between oocyte (including zona) measurements and oocyte maturation in vitro.

Figure 7.22 shows the relationship between the oocyte (including zona) diameter and the ability to fertilize and cleave. Figure 7.22a shows that 2/6 (33%) of oocytes that matured after 23-24 hr of in vitro culture cleaved following fertilization, whereas 5/6 (83%) of oocytes maturing after 46-48 hr cleaved following fertilization (Figure 7.22b). The smallest oocyte to cleave following fertilization had a diameter (including zona) of 152μm on day 0, however the median oocyte diameter (including zona) on day 0 for oocytes that fertilized and cleaved was 161μm, interquartile ranges = 157.7-162, n = 12, range = 152-174.

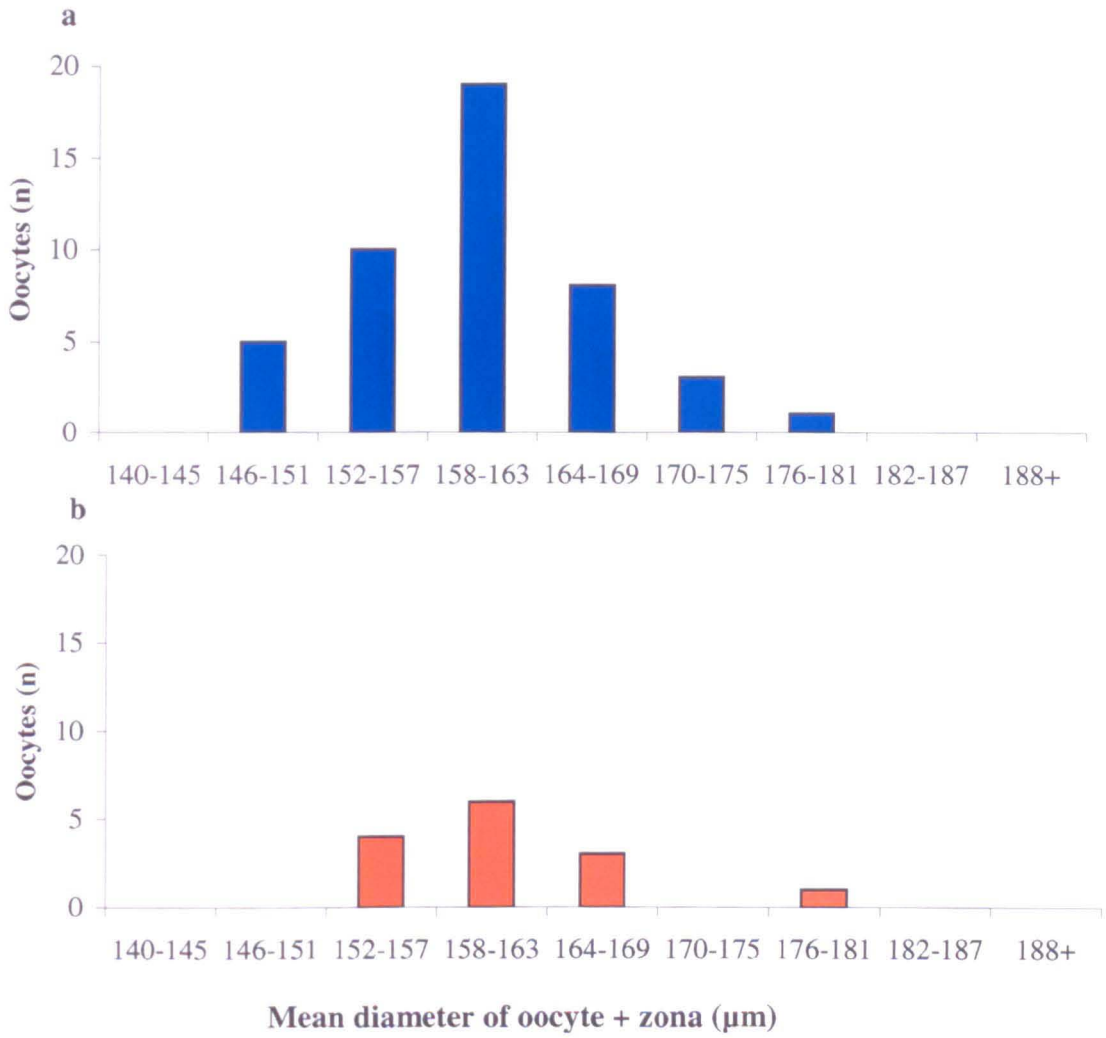


Figure 7.20 Frequency histogram showing mean oocyte diameters (including zona) on day of collection of oocytes from patients undergoing ICSI treatment.
a) Viable oocytes
b) Oocytes becoming mature in vitro

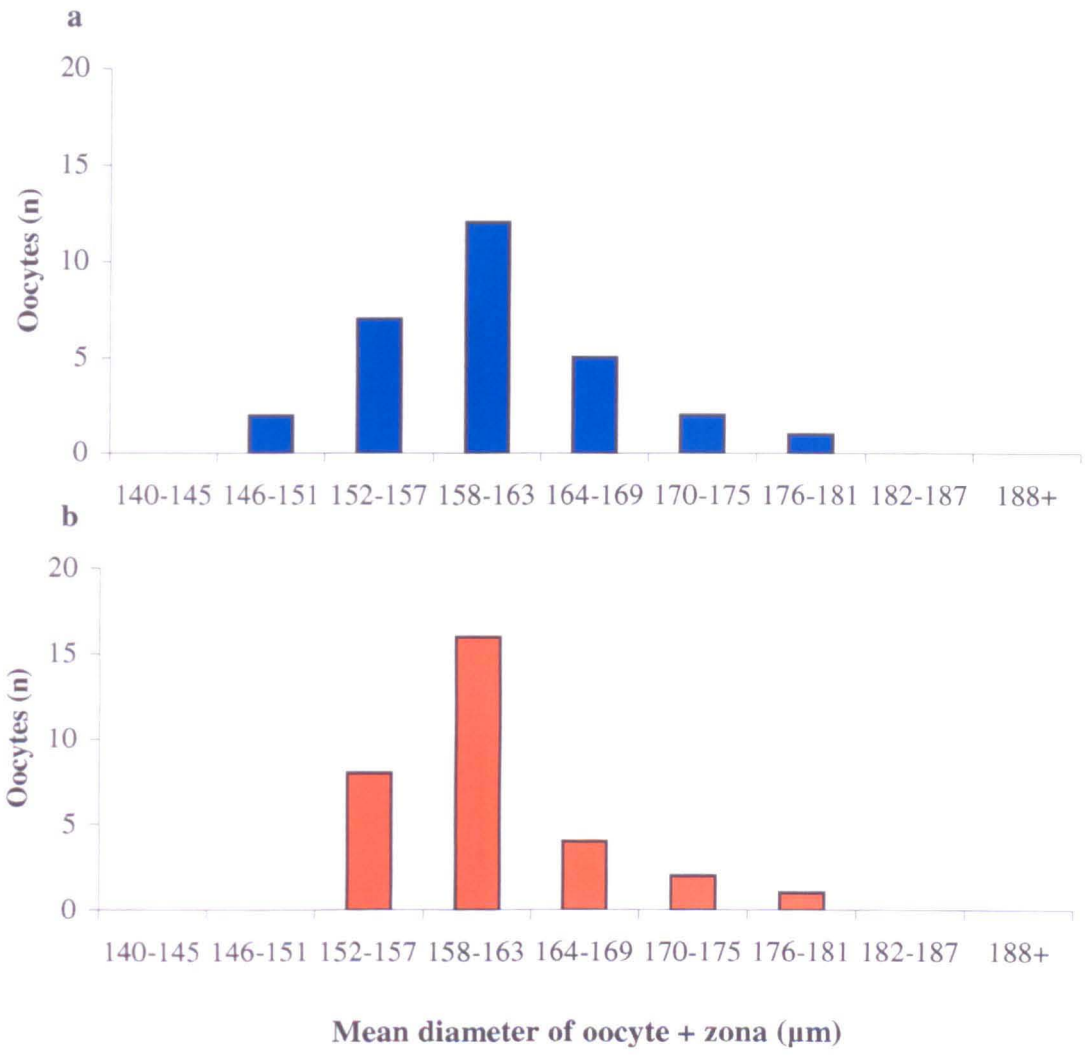


Figure 7.21 Frequency histogram showing mean oocyte diameters (including zona) on day of ICSI (day 0) of oocytes from patients undergoing ICSI treatment.
a) Immature or atretic oocytes
b) Mature oocytes

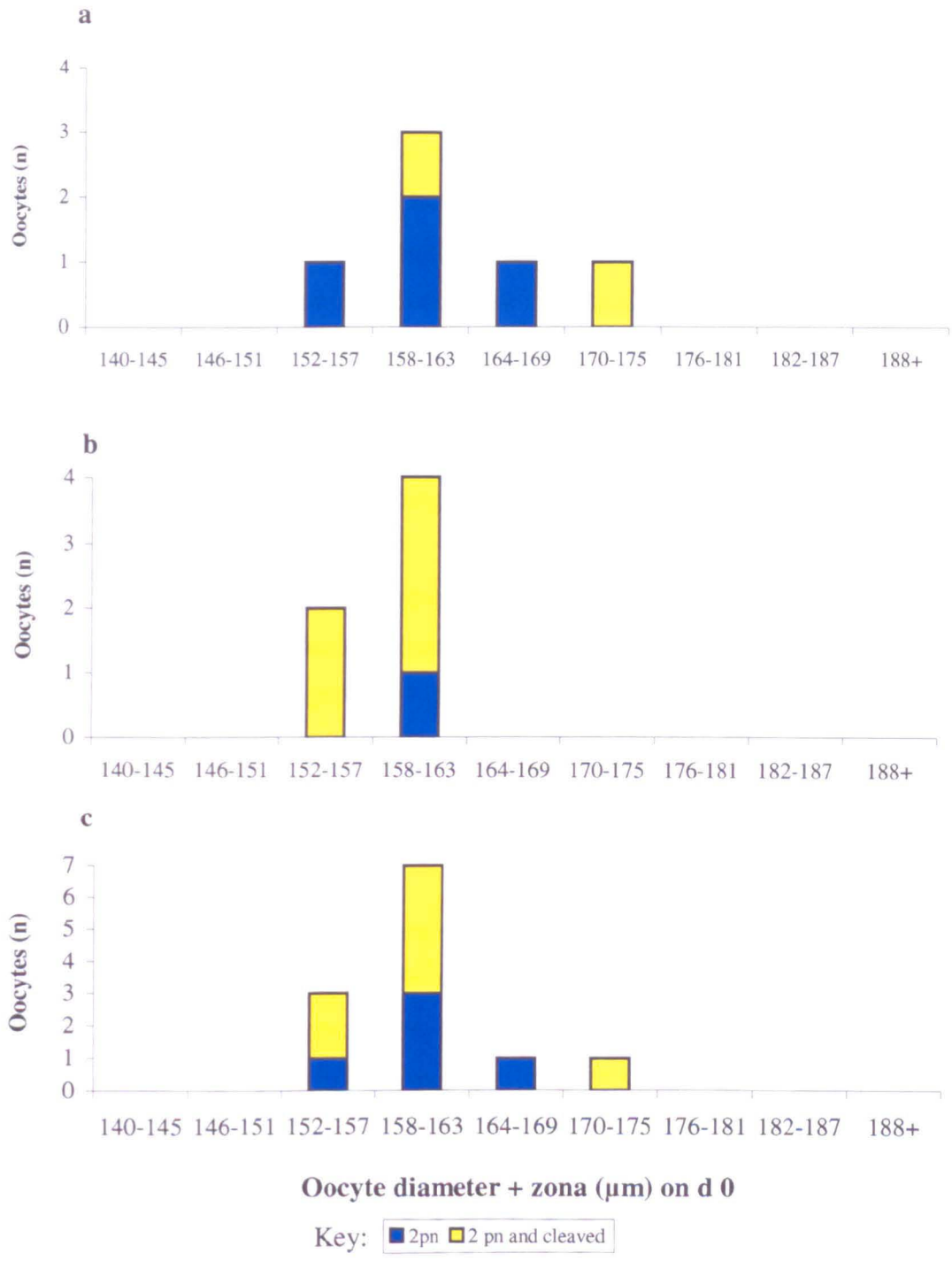


Figure 7.22 Frequency histogram of fertilized oocytes obtained from patients undergoing ICSI treatment, undergoing cleavage or arresting at the 2pn stage according to the oocyte + zona diameter on day 0.
a) Oocytes that matured in 23-24 hr
b) Oocytes that matured in 46-48 hr
c) Oocytes that fertilized or fertilized and cleaved, irrespective of the time taken to mature in vitro

7.3.3 Zona pellucida thickness

Figure 7.23a shows that the mean zona pellucida thickness on the day of collection of viable oocytes donated by patients undergoing ICSI treatment was not normally distributed, in contrast to similar data obtained for oocytes derived from PCO patients (Figure 7.9). The most common size range was 21-22μm (27%). Similar proportions of oocytes having different zona pellucida thickness measurements matured in vitro; hence there was no apparent relationship between zona pellucida thickness on day of collection and the subsequent maturation of the oocyte. The median and range values of zona pellucida thickness were similar for oocytes from patients with PCO and those undergoing ICSI treatment on the day of collection.

Figure 7.24 shows the frequency histogram of zona pellucida thickness of oocytes on day 0 according to whether they matured in vitro. Between the day of collection (Figure 7.23) and day 0 (Figure 7.24) slight alterations in the distribution of zona pellucida thickness were evident. However, there was no relationship evident between zona pellucida thickness on day 0 and oocyte maturation in vitro (Figure 7.24).

Figure 7.25 shows the relationship between time and zona pellucida thickness during in vitro culture with and without FF-MAS. Changes in zona pellucida thickness were not related to the presence or absence of FF-MAS. However, the zona pellucida thickness appeared to be more consistent in oocytes obtained from patients undergoing ICSI (Figure 7.25) compared to oocytes obtained from patients with PCO (Figure 7.11). The oocytes obtained from patients with PCO were smaller than those obtained from patients undergoing ICSI treatment on the day of collection and so it may be that the zona pellucidas on the smaller oocytes from patients with PCO were still forming or growing and changing in vitro.

Table 7.6 shows the zona pellucida thickness on day +1 and day +2 of oocytes collected from patients undergoing ICSI treatment, that fertilized and those that failed to fertilize or became atretic. There was no significant difference in zona pellucida thickness between day +1 and day +2 in either group, nor between the groups of oocytes that did or did not fertilize. This is in contrast to the data in table 7.5, which shows that the fertilized oocytes arising from in vitro matured oocytes,

collected from patients with PCO tended towards significantly thicker zonas ($p<0.05$).

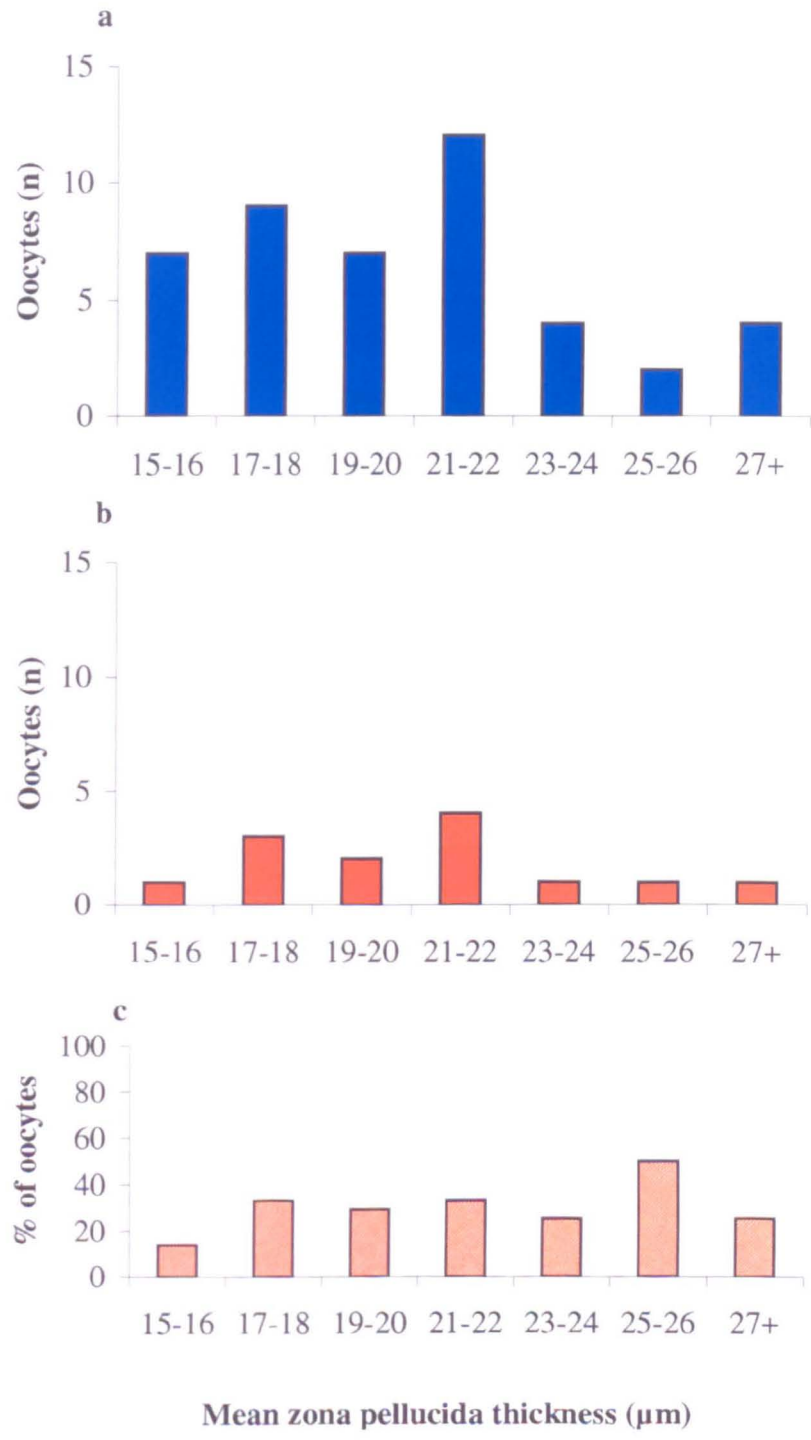


Figure 7.23 Frequency histogram showing mean zona pellucida thickness on day of collection of oocytes from patients undergoing ICSI treatment
a) Viable oocytes
b) Oocytes becoming mature in vitro
c) Oocytes that progressed to maturity expressed as a percentage of all viable oocytes on day of collection

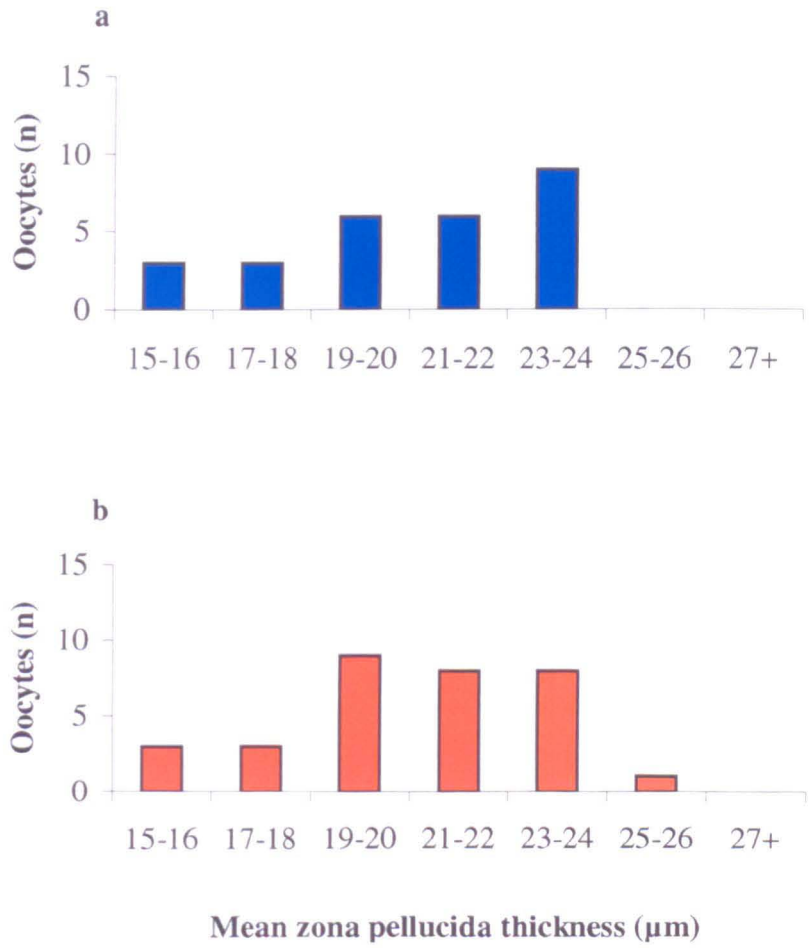


Figure 7.24 Frequency histogram showing mean zona pellucida thickness on day 0 (day of ICSI for mature oocytes) of oocytes from patients undergoing ICSI treatment.
a) Immature or atretic oocytes
b) Mature oocytes

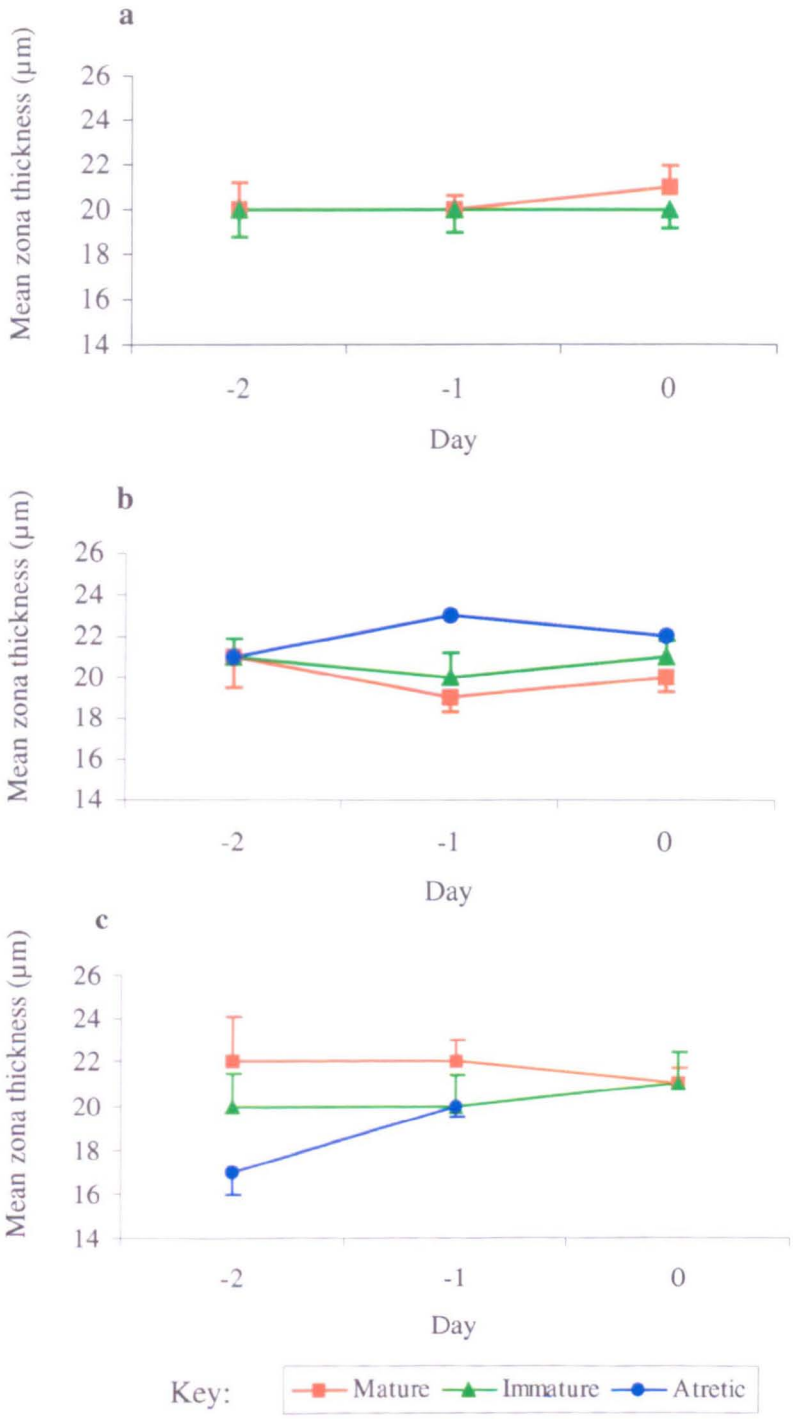


Figure 7.25 Relationship between time and zona pellucida thickness during in vitro culture in a) control, b) 10µg/ml FF-MAS and c) 30µg/ml FF-MAS. These oocytes were collected from patients undergoing ICSI treatment. Results are expressed as mean ± SEM.

Table 7.6 Zona pellucida thickness on day+1 and day+2 of oocytes collected from patients undergoing ICSI treatment, according to whether they fertilized or failed to fertilize/became atretic

	Mean zona pellucida thickness (μm)	
	Day+1	**Day+2**
Fertilized	20.5 ± 0.8	21.2 ± 0.9
Range	17.8-25.1	18.1-24.6
Unfertilized/atretic	20.3 ± 0.6	19.3 ± 0.5
Range	17.9-22.0	18.9-19.8

Mean ± SD

7.3.4 Perivitelline space (PVS)

Figure 7.26a presents the frequency histogram of the mean PVS of viable oocytes collected from patients undergoing ICSI treatment. The majority of oocytes (82%) had a mean PVS between 3-6μm on the day of collection. Most measurements on the day of collection, of oocytes that became mature in vitro were also between 3-6μm (Figure 7.26b).

Figure 7.27 shows the frequency histogram of the mean PVS of oocytes from patients undergoing ICSI treatment on day 0 (the day of ICSI of mature oocytes) according to whether the oocytes matured in vitro. The distribution of PVS was similar in oocytes that matured in vitro and in those that remained immature or became atretic.

Figure 7.28 shows the relationship between time and mean PVS during in vitro culture with and without FF-MAS. The data do not indicate any relationship between PVS and oocyte maturation in vitro, and the inclusion of FF-MAS in the media seems unrelated to this measurement. There was no significant variation with time, of the PVS thickness of mature and immature oocytes throughout the culture period.

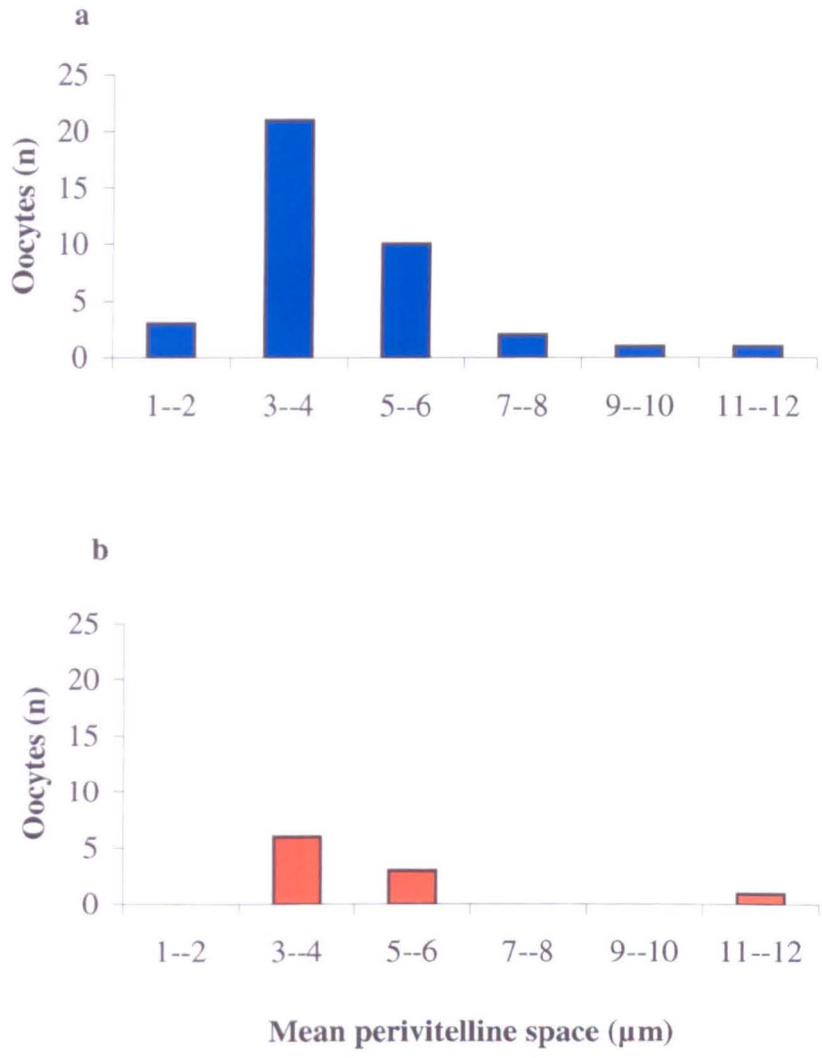


Figure 7.26 Frequency histogram showing mean perivitelline space on day of collection of oocytes from patients undergoing ICSI treatment.
a) Viable oocytes
b) Oocytes becoming mature in vitro

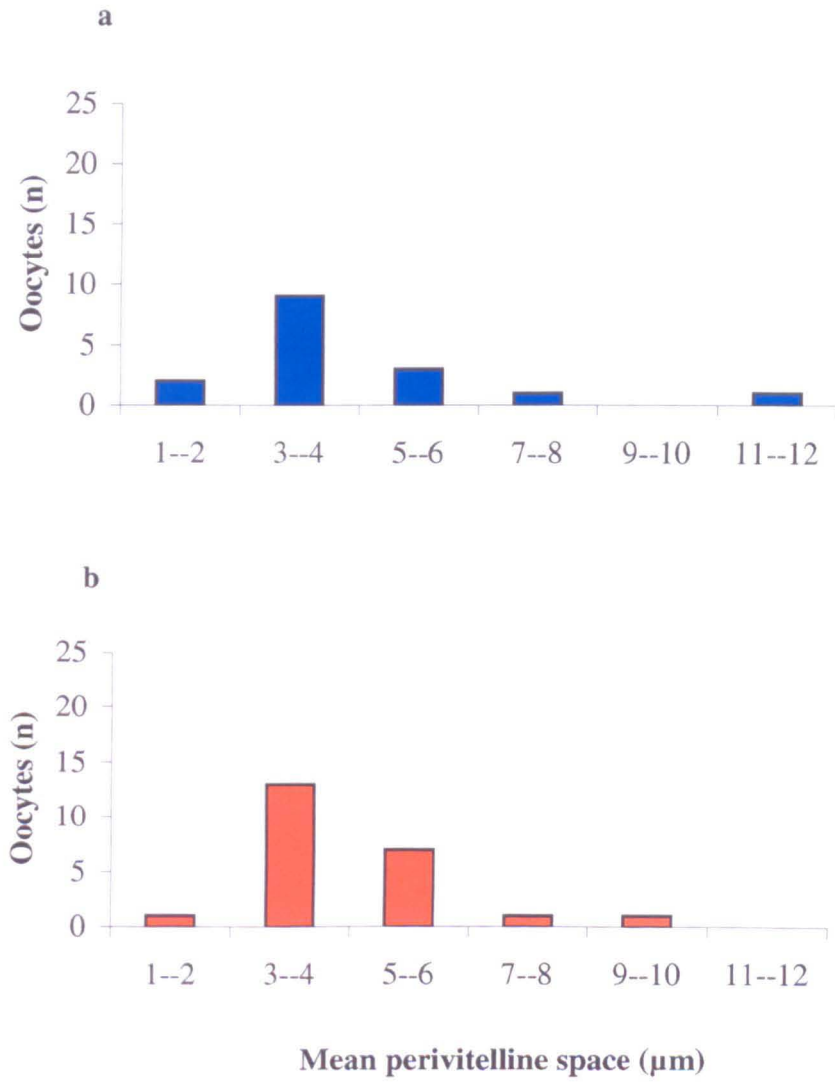


Figure 7.27 Frequency histogram showing mean perivitelline space on day 0 (day of ICSI for mature oocytes) of oocytes from patients undergoing ICSI treatment.
a) Immature or atretic oocytes
b) Mature oocytes

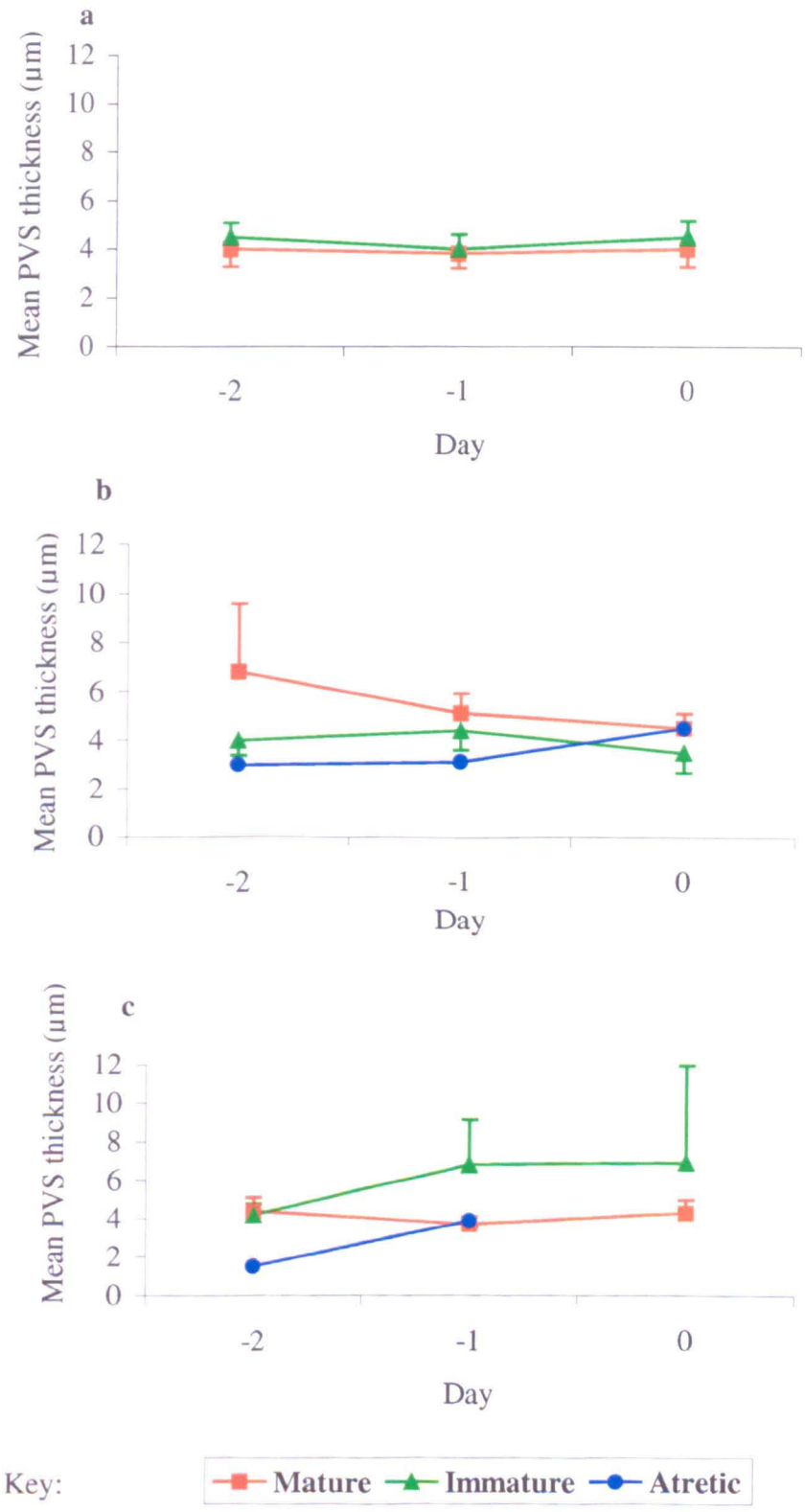


Figure 7.28 Relationship between time and mean perivitelline space (PVS) during in vitro culture in a) control, b) 10µg/ml FF-MAS and c) 30µg/ml FF-MAS collected from patients undergoing ICSI treatment. Results are expressed as mean ± SEM.

7.4 Discussion

Oocyte development incorporates two major activities: growth and maturation. Throughout the growth phase, the oocyte is arrested in prophase of the first meiotic division but increases in diameter from ~30μm to ~120μm, which is associated with a 470-fold increase in volume. The duration of the growth phase of the human oocyte is estimated to be ~8-12 weeks (Gougeon, 1986) compared to three weeks in the mouse (Carroll, 1996).

The diameter of the mature human oocyte excluding the zona pellucida is normally approximately 110-120μm; the zona pellucida is normally approximately 15-20μm thick and becomes slightly thinner after fertilization, although zona pellucida anomalies including excessive thickness are quite commonly noted in IVF and may be patient or maturity related. Including the zona pellucida and perivitelline space, the pre-ovulatory oocyte commonly has a diameter of approximately 150μm (Veeck, 1999). Oocytes that are much larger than normal, sometimes up to twice the volume of normal oocytes, are presumed to have arisen through failure of cytoplasmic division of oogonia. Most of these oocytes possess diploid amounts of DNA and some are binucleate. After penetration of a binucleate oocyte by a single spermatozoon, two maternal pronuclei and one paternal pronucleus may be formed, resulting in triploidy (Veeck, 1999).

My project is the first to monitor the growth of human oocytes during in vitro maturation, and has produced some interesting results showing that measurable oocyte growth may continue in vitro and may relate to the eventual outcome of maturation and insemination in vitro. Moreover, the starting material obtained from the two different patient populations was significantly different in oocyte diameter, which may help to explain observations on the different results observed in this study (Chapter 5) and by previous authors, according to the origins of the oocytes.

Durinzi *et al.* (1995) examined the relationship between oocyte size and maturation in vitro in unstimulated human oocytes. They set out to determine if the size of human oocytes at collection is related to their ability to resume meiosis and undergo maturation in vitro. They observed a significant difference in oocytes measuring 86-

105μm versus those measuring 106-125μm, leading to the conclusion that, in common with other species (see introduction, 7.1) the unstimulated human oocyte has a size-dependent ability to resume meiosis and complete maturation. The following oocyte diameter thresholds in relation to meiotic potential were deduced: oocyte diameter ≤ 105μm: GV; oocyte diameter > 105μm < 115μm: GVBD → MI; oocyte diameter > 115μm: GVBD → MII. My data for oocytes retrieved from patients with PCO produced a threshold of ~103μm for maturation in vitro, similar to Durinzi *et al.* (1995), however oocytes were observed to have reached meiotic maturity in vitro with an oocyte diameter considerably less than 115μm. However, interestingly, none of the oocytes from stimulated patients undergoing ICSI treatment, and having initial diameters of ≥115μm matured in vitro. In my study, measurements of oocyte diameter on the day of oocyte recovery and the day of insemination for mature oocytes both demonstrated size dependent maturation, as well as the capacity for oocyte growth within this time frame.

In 1977, Moor and Trounson analysed the developmental potential of fully grown ovine oocytes retrieved from antral follicles of known sizes, it was established that follicles had to be >2-3mm diameter to confer developmental competence on ovine oocytes, these results have been confirmed in a variety of species by showing that oocytes become competent only when follicles reach diameters ranging from ~500μm in mice to 3-4mm in cattle (Eppig *et al.*, 1992; Pavlock *et al.*, 1992; Lonergan *et al.*, 1994; Blondin and Sinard, 1995). Dubey *et al.* (1995) suggested that competence in human oocytes may normally be conferred relatively late; perhaps only when follicles have reached diameters of >10mm, although occasional pregnancies have resulted from IVM of oocytes from smaller follicles (Trounson *et al.*, 1994). The percentage of maturing human oocytes from non-stimulated ovaries increases with follicular size (3-15mm) during the follicular phase but appears to remain constant during the luteal phase, irrespective of follicle size (Tsuji *et al.*, 1985). In the follicular phase, the proportion of oocytes maturing in the large follicle group (9-15mm diameter; 34.5%) was significantly ($p < 0.05$) higher than that in the small follicle group (3-4mm; 8.8%).

In my study, oocytes from patients with PCO were retrieved from antral follicles approximately 10mm diameter or less, whilst oocytes donated by patients undergoing ICSI were retrieved from larger follicles >10mm diameter, although other differences may have contributed, as well as follicle size. Oocytes retrieved from patients undergoing ICSI were significantly larger at collection (mean oocyte diameter 111μm) than oocytes retrieved from patients with PCO (106μm), which may have been partially due to the larger size of follicles in patients undergoing ICSI. It was more difficult to measure oocyte diameter for those where a substantial covering of cumulus obscured the oolemma, so it is possible that oocytes with a dense cumulus cover were a different size to those with less cumulus. However, those oocytes with a dense cumulus cover that could be measured (Table 7.4) were not significantly larger than those with less or no cumulus cover, so it is probable that these results can be generalised.

Mature, immature and atretic oocytes from patients with PCO cultured in FF-MAS culture conditions (10 or 30μg/ml) but not those cultured in control conditions were significantly different in terms of diameter on day 0 ($p < 0.05$), though not on day-2. Similar results did not occur for oocytes donated by patients undergoing ICSI treatment. The reason for this is not clear. However in the oocytes from patients undergoing ICSI, there was a significant enlargement of oocyte diameter between the day of collection and day 0 in both FF-MAS culture conditions, although not in control, indicating significant growth of oocytes in vitro with FF-MAS. Therefore, a response to FF-MAS in terms of oocyte growth, a quantifiable, non-invasive parameter, was evident in immature oocytes from both sources of patients. As oocytes from both patient groups have grown in vitro, it is clear that the growth phase has not been completed in vivo, or that it may be resumed under certain conditions. In 1998, Moor *et al.* suggested that the reduced developmental potential in human oocytes matured in vitro might be attributable to incomplete oocyte growth, however, no data were presented on human oocytes to illustrate this suggestion.

Karnikova *et al.* (1998) suggested that a critical nucleocytoplasmic volume ratio is necessary for normal maturation in mammalian oocytes. They demonstrated that a reduction in cytoplasmic volume before culture of fully-grown mouse oocytes isolated

from large antral follicles and cultured in vitro, resulted in a decrease in the proportion of oocytes completing maturation and extruding a pb. The time course of GVBD was also delayed in relation to the decreased cytoplasmic volume. However, there may be many reasons for this, such as a reduced number of cytoplasmic organelles, or a reduced surface area for metabolic exchange.

In my study, among oocytes that matured in culture from patients with PCO, 43% had a diameter >109μm on day 0 compared to 21% in this size range among those not maturing, which was significantly different ($p<0.05$), therefore oocytes with a smaller diameter appear to be less likely to mature, however the same was not true for oocytes retrieved from patients undergoing ICSI. The reason for this discrepancy might relate to the prior exposure of oocytes to ovarian stimulation, which may have supported their growth in vitro while not facilitating their meiotic maturation. Clearly, then, in some respects oocytes from patients with PCO and those undergoing ICSI are behaving differently under similar conditions, (e.g. growth) whereas in other respects, they are different populations (e.g. size dependence). For both patient groups, and in all culture conditions the diameter of oocytes that underwent atresia in culture decreased during the culture period. This is likely to be attributed to oocyte shrinkage commonly associated with atresia. The differences in oocyte diameter after IVM may be linked to changes in osmolarity of the cytoplasm, potentially linked to membrane properties or some other factor. In this study, control experiments, (Chapter 2, section 2.2.1.1) demonstrated that the osmolarity of cultures without an oil overlay maintained in a humidified incubator (37°C, 5% CO_2 in air) varied by <1% after 24 hr. However, both increases (maturing) and decreases (atresia) in oocyte diameter were observed under similar conditions in the same culture preparations in these experiments, discounting alterations in media osmolarity as the mechanism by which oocyte size changes occurred.

FF-MAS is a steroid related to cholesterol, and cholesterol is known to influence membrane fluidity. It is possible that FF-MAS, or another steroid component of the system associated with oocyte maturity may have a direct effect upon membrane fluidity or function, which might contribute to the effects observed in these experiments.

The perivitelline space (PVS) is the area between the inner surface of the zona pellucida and the oolemma. The polar bodies are released from the oocyte into the PVS, as are the contents of the cortical granules in the zona reaction. This study has demonstrated a variation in thickness of the PVS of oocytes after IVM; however there did not appear to be a pattern of shrinkage or growth of the PVS during in vitro culture with or without MAS, or in association with the maturation of the oocyte. Oocyte shrinkage is associated with atresia and this might be expected to enlarge the PVS (Hyttel *et al.*, 1986), however this did not occur in a consistent fashion in this study. Overall, the PVS measurements were variable and inconsistent. This could be a result of single plane measurements on spherical objects, as well as the low proportion of reliable measurements of this feature that could be achieved in this study.

The zona pellucida is a structure that is crucial to fertilization and early development. It is synthesized by the oocyte and secreted to form an extracellular protein matrix that surrounds the growing oocyte, ovulated oocyte and preimplantation embryo (Dean, 1991). According to Bertrand *et al.* (1995), human zona pellucida thickness varies from 10-31μm, with a mean of 17.5μm. A zona pellucida thickness of >25μm or <10μm is rarely observed. The zona pellucida thickness is a feature of individual oocytes that influences sperm penetration (Bertrand *et al.*, 1995,1996), and ICSI is recommended in order to encourage fertilization of oocytes with particularly thick zonas. In this study, on day 0 all mature oocytes had a zona thickness measurement of between 15-24μm, which is within the expected range. Zona measurements were normally distributed but appeared not to be related to maturity.

In 1996, Bertrand *et al.* studied the zona pellucida thickness of 827 human oocytes 16-20 hr after in vitro insemination. The zona pellucida of fertilized oocytes (16.6 ± 3.2μm) was significantly thinner than that of unfertilized oocytes (18.9 ± 4.0μm; $p< 0.001$), but this was not related to ooplasm diameter. Thinning of the zona pellucida is believed to continue during the first and second cleavages (Veeck, 1999). However, my data do not follow the same pattern.

The increased size of oocytes + zona for patients undergoing ICSI treatment on day of collection is consistent with the larger oocyte diameter at collection for this patient

group. The oocyte plus zona measurements did not offer any additional information to oocyte diameter for either patient groups, and may have reduced the discriminatory potential of oolemma measurements. The zona pellucida would not be expected to change size greatly during a short period of culture, however, clearly changes in size and/or thickness have to be accommodated during the oocyte's growth phase.

It has been suggested that zona hardening may occur during maturation in vitro (Barnes *et al.*, 1996; Cha and Chian, 1998) and ICSI has been shown to increase fertilization rates of in vitro matured oocytes from 30% for routine insemination by sperm incubation in vitro to 50% (Trounson *et al.*, 1996).

Garside *et al.* (1997) suggested that zona pellucida measurements should be included in the overall assessment of human embryo quality, as it may be useful in the selection of optimal embryos for transfer. They used an ocular micrometer to measure zona pellucida thickness in zygotes and cleavage stage embryos daily during in vitro culture. They found that the average zona thickness decreased during culture after fertilization, from 17.7 ± 0.14 μm on day 1 of in vitro culture to 16.3 ± 0.14 μm on day 2 and 14.9 ± 0.14μm on day 3 ($p < 0.0001$). Embryos transferred and resulting in a successful pregnancy cycle had significantly thinner zona pellucidas ($p < 0.0001$) when compared to embryos that were not transferred or from non-conception cycles. They also demonstrated a decrease in average zona thickness with age, most apparent after 35 years. Loret De Mola *et al.* (1997) analyzed the thickness of the human zona pellucida during culture, and also suggested that zona pellucida thickness be considered as part of the embryo quality evaluation prior to transfer or when assessing the possibility of using assisted hatching. They hypothesized that zona pellucida thickness is influenced by the preovulatory hormonal environment and infertility diagnosis.

Cohen *et al.* (1989) and Gabrielsen *et al.* (2000) studied the influence of zona pellucida thickness of human embryos on clinical pregnancy outcome following IVF treatment. Cohen *et al.* (1989) concluded that an increased variation of zona pellucida thickness was the most important prognostic factor for fresh embryo implantation. When the "best" embryo had > 25% zona pellucida thickness variation (ZPTV), 40 %

(24/60) resulted in pregnancy, however when the "best" embryo had <10 % ZPTV pregnancies were not induced (0/21). Gabrielsen *et al.* (2000) observed a highly significant correlation between ZPTV of transferred embryos and the IVF outcome, with 84% of the clinical pregnancies resulting from transferred embryos with ZPTV values greater than 25%. The mean ZPTV values for 70 conception cycles and 62 nonconception cycles were 28 ± 6.4 and 17.8 ± 8.1 respectively. They suggest that ZPTV could be used as a criterion for embryo selection during clinical transfers.

The measurements of zona thickness of fertilized oocytes on day+1 from both patient groups in this study appeared significantly larger than the data (measurements of in vivo matured oocytes) of Garside *et al.* (1997). On day +1, fertilized oocytes obtained from patients with PCO and patients undergoing ICSI treatment had mean zona pellucida thickness measurements of 21.8 ± 1.9μm and 20.5 ± 0.8μm respectively, compared to 16.4 ± 3.1μm (Bertrand *et al.,* 1996) and 17.7 ± 0.14μm (Garside *et al.,* 1997). On day +2, fertilized oocytes obtained from patients with PCO and patients undergoing ICSI treatment had mean zona pellucida thickness measurements of 20.1 ± 2.9μm and 21.2 ± 0.9μm respectively, compared to 16.3 ± 0.14μm (Garside *et al.,* 1997). Zona pellucida thickness appeared to change to some extent during culture in my study. The reasons for this difference are unclear but could relate to differences in methodology and/or starting material, including the origin of the oocytes. In addition, some effect of IVM cannot be ruled out at this stage.

Overall, this novel study has provided the first quantitative non-invasive analysis of human oocyte growth during maturation under various conditions in vitro. It has laid the foundations for the application of a non-invasive assessment of oocyte maturation potential in vitro, which will be an advantage for the future clinical use of IVM where selection of the two embryos with the greatest potential for development is the key to a successful outcome. This is particularly true of in vitro matured oocytes, where developmental potential is known to be a limiting factor. Such quantitative methods could also be applied in other areas such as zygote analysis and assessment of the regularity of early cleavage, where eventually, their incorporation into an automated system of oocyte and embryo monitoring might be feasible.

Chapter 8

General Conclusions

The aim of this project was to assess specific factors affecting IVM of human oocytes in relation to the developmental competence of the resulting embryos. This task was undertaken using a variety of approaches, to study immature oocytes arising from two patient groups, those with unstimulated PCO undergoing treatments which required a laparoscopy; and those undergoing ICSI treatment, which included ovarian stimulation according to a standard 'long' pituitary down-regulation protocol. As well as providing original information on oocyte responses to supplementation of maturation medium in vitro, this project has provided an opportunity to develop technical expertise, which will be essential to the eventual clinical application of IVM in this country.

Chapter 3 presented the characteristics of patients donating oocytes to this study, in order to assess whether particular patients or groups might be more or less suitable for immature oocyte collection and IVM. Clear differences between the patient groups were noted, as expected, and this allowed the subsequent experiments to be conducted on oocytes originating from contrasting endocrine environments. While differences between the two patient groups were evident, both later demonstrated positive effects of manipulation of the IVM procedure, suggesting that clinical IVM might potentially be applicable to both types of patients.

Two factors were investigated for their effects upon IVM and subsequent development of immature oocytes. These were selected based upon previous work suggesting their potential to promote embryonic development through effects mediated during oocyte maturation. The effect of supplementation of the IVM culture medium with EGF was investigated in human immature oocytes obtained from unstimulated ovaries from women with PCO. The EGF concentrations used (0.1-10 ng/ml) were similar to those shown to be effective in previous studies (Das *et al.*, 1991; Gomez *et al.*, 1993; Goud *et al.*, 1998). Any mature oocytes arising from in vitro culture were inseminated with known fertile donor sperm using ICSI in order to assess any effects of EGF on fertilization, cleavage and embryo development. Data presented in Chapter 4 did not generally indicate significant effects of EGF used

alone, in the absence of gonadotrophins, on in-vitro survival and maturation of human oocytes. This was in contrast to data provided by Das *et al.* (1991), Gómez *et al.* (1993a), and Goud *et al.* (1998) who had all reported beneficial effects of EGF in human IVM. However, the patient groups and culture conditions varied from those in this study. When the developmental competence of the matured oocytes in terms of their fertilization and embryo development after maturation with EGF was assessed, no significant effect of EGF on fertilization and cleavage was observed possibly due to the low numbers of oocytes. However, cleaving embryos were achieved in all culture conditions confirming that the oocytes matured in vitro were capable of fertilization and the initial stages of embryo development, despite the absence of gonadotrophin stimulation in vitro.

Human oocyte availability is a limiting factor in such research; hence the use of EGF for IVM was not studied further. Instead, the more promising results on the use of FF-MAS, the putative physiological mediator of oocyte maturation in mice (Byskov *et al.*, 1995, 1999; Hegele-Hartung *et al.*, 1999), were pursued with the first study of this compound on human oocytes. This was and remains a new and exciting area, since no data on this subject had been previously published and there may be many benefits of applying the proposed physiological effector rather than highly potent purified growth factors, whose unopposed effects might eventually prove hazardous for development. To date, little is known about the precise mechanism of FF-MAS action in human oocyte maturation in vivo, although it is found in high concentrations in follicular fluid just prior to final oocyte maturation and ovulation (Byskov *et al.*, 1999; Grondahl *et al.*, 2000). This study has contributed to the literature on this topic and presents the largest series studied to date (Cavilla *et al.*, 2001).

The study design and results presented in chapter five allowed not only an assessment of the effect of FF-MAS exposure during IVM of human oocytes upon their maturation, fertilization and early embryonic development, but also enabled a comparison of the results of FF-MAS exposure in immature oocytes originating from two different patient groups. The data demonstrated that FF-MAS at a concentration of 30μg/ml significantly ($P < 0.025$) increased oocyte survival resulting in 88%

survival compared with 63% and 60% for 10μg/ml and control respectively in the unstimulated patient group, although the proportion of oocytes completing maturation in vitro was not significantly altered. In contrast, for the patients undergoing ICSI treatment, oocyte survival was >90% in all groups, regardless of FF-MAS concentration. However, in this patient group, the presence of FF-MAS at 10 or 30μg/ml significantly increased the proportion of oocytes maturing ($P < 0.05$). The time course of oocyte maturation differed between the two patient groups, with oocytes from patients undergoing ICSI maturing more rapidly in vitro than those from patients with PCO, most likely as a result of their differing follicular environments prior to oocyte collection. In terms of further development, in both patient groups, oocytes cultured with FF-MAS showed a tendency towards improved subsequent development, which, though insignificant with the numbers of oocytes and embryos available, may be an important observation for future application, since poor developmental potential of in vitro matured oocytes currently remains an intractable problem.

Among the oocytes obtained from patients with PCO, those with attached cumulus cells had a greater tendency towards survival and maturation in vitro than nude oocytes in all culture conditions. Overall, the rates of IVM achieved in this study were acceptable, but not outstanding in comparison with publications where oocyte maturation was achieved under different conditions. There may be a variety of reasons for this. For example, a higher maturation rate may have been gained by a combination of supplements in the medium; for example, addition of FSH may have augmented FF-MAS production in the cumulus (Byskov *et al.,* 1997) and the inclusion of FSH, HCG and/or other growth factors in vitro has previously been demonstrated by others to stimulate IVM (Durinzi *et al.,* 1997; Chian *et al.,* 1999a,b). However, these factors were omitted here in order that the effects of FF-MAS alone could be established in the absence of confounding influences.

Especially high levels of atresia occurring in vitro after collection of apparently viable oocytes were noted in GVBD oocytes from PCO patients. The incidence of atresia was significantly higher in GVBD oocytes than GV oocytes collected from PCO patients ($p < 0.001$). Compromised follicle development as a result of the abnormal

endocrine environment could account for the higher rate of atresia observed in the GVBD oocytes from patients with PCO compared with the ICSI patient group. In the oocytes obtained from patients undergoing ICSI treatment, atresia was confined to oocytes collected at the GV stage. Overall the oocytes obtained from patients with PCO appeared more susceptible than oocytes obtained from patients undergoing ICSI treatment however, patients having PCO are amongst those who may gain most from the potential clinical application of IVM, yielding a larger cohort of oocytes than non-PCO patients (Trounson *et al.,* 1996) and being the most likely to suffer hyperstimulation as a result of gonadotrophic drug exposure in vivo (Rizk and Smitz, 1992). Hence, continuing research into IVM in this group of patients is of particular value. Research in cattle has shown that oocytes arising from follicles in the early stages of atresia are as competent, or even more so, to initiate embryonic development than oocytes from actively growing follicles (Sirard and Blondin, 1996). Follicles in early atresia may contain competent oocytes, whereas oocytes from follicles at advanced stages of atresia may contain oocytes less competent to develop into viable embryos (Hendriksen *et al.,* 2000; Mikkelsen, 2001). The endocrine environment of follicles in patients with PCO may contribute to premature oocyte maturation, however appropriate control of this situation in future, perhaps by gonadotrophic manipulation following a withdrawal bleed, may offer potential gains for IVM application in this group of patients.

IVM is widely reported to result in embryos of reduced developmental competence relative to those undergoing oocyte maturation in vivo. The only true assessment of the success of IVM is live birth. This most stringent measure of developmental competence of embryos can rarely be applied in human IVM research, in which the transfer of embryos subjected to research procedures is potentially risky and is constrained by legal and ethical concerns. The transfer of in vitro matured and fertilized oocytes is not yet permitted in the UK and the HFEA required that a research study, in which embryos are not transferred to patients, must be conducted prior to any application for a clinical licence. Hence, it remains unclear whether the embryos resulting from in vitro maturation in this study might have had the potential for complete developmental competence if transferred to the uterus, however, their in vitro development did not progress beyond cleavage.

While FF-MAS was shown by my work to have positive effects on maturation in vitro, and possibly subsequent development, the normality of the resulting gametes had not been assessed directly. This presented some concerns since a large proportion (34-50%) of human oocytes are known to be abnormal (Martin *et al.,* 1986; Wramsby *et al.,* 1987) and no test for "normality" currently exists. Maturation comprises two major aspects, genetic (meiotic) maturation and cytoplasmic maturation (Fulka *et al.,* 1998). These two areas were tackled and the potential effects of FF-MAS explored in so far as was possible, however, the lack of fresh, good quality, in vivo matured oocytes as a control group causes difficulties in interpretation, most notably of genetic maturation where in vitro ageing of gametes is known to influence competence adversely. In Chapter 6, a preliminary study was undertaken to study the maturing oocytes' chromosomal constitution. This assessment used oocytes from patients undergoing ICSI only, this being the most readily available source of human oocytes and less likely to suffer from the unknown effects of the PCO endocrine environment, having been exposed to a controlled regime of gonadotrophic stimulation. Based upon the rather disappointing results, three analysable spreads from 15 oocytes; this line of investigation was discontinued in favour of studying spindle appearance and chromosome alignment using fluorescent markers for tubulin and chromatin.

In 1999, Hegele-Hartung *et al.* observed the formation of a barrel shaped spindle and apparently normal chromosome alignment in FF-MAS treated mouse oocytes. In contrast, the findings of my preliminary study of spindle formation during IVM with exposure to FF-MAS demonstrated major abnormalities in the chromosome distribution upon the oocytes' spindles after IVM and abnormalities in spindle development. These may possibly be major contributors to the limited developmental potential of oocytes matured in vitro observed in this series and reported by others (Barnes *et al.,* 1996; Trounson *et al.,* 1996, Hu *et al.,* 2001). However, the extent to which the abnormal spindle formation and chromosome alignment was a reflection of IVM or FF-MAS exposure is unclear, because control oocytes rarely matured in the absence of FF-MAS. The absence of gonadotrophins in vitro, the natural stimulus for meiotic maturation via the midcycle surge in vivo, may also have affected this analysis.

Aberrations in the cytoplasmic maturation of oocytes are more likely to be apparent as failure in later stages of development than in failure to reach metaphase II arrest. A non-invasive technique for assessing developmental competence is not currently available but would be a major advantage in human assisted conception and particularly in the application of IVM, where developmental competence is lower than for in vivo matured oocytes. In Chapter 7, a large database of images was analysed, enabling quantifiable morphological features to be related to the in vitro culture conditions employed and/or the subsequent development of the oocyte/embryo. The use of image analysis provided a non-invasive technique by which to compare and study maturing oocytes and this study, which is in preparation for publication, has laid the foundations for a non-invasive assessment of developmental competence during maturation in vitro. The presence of residual cumulus cells limited the feasibility of taking measurements from the whole image set, however, further refinements of the laboratory and imaging techniques could overcome some of these problems in future, if this procedure's usefulness in non-invasive assessment is proven clinically.

Oocyte size is associated with maturation, fertilization and embryo development in most species examined and this may indicate that a certain absolute volume is necessary to initiate the molecular cascade of normal nuclear and cytoplasmic maturation (Trounson *et al.,* 2001). During preantral follicle growth the diameter of the human oocyte increases markedly from approximately 35μm to 120μm, equivalent to a 470-fold increase in volume, crossing the threshold for meiotic competence at about 109μm diameter (Durinzi *et al.,* 1995). An increased ability to undergo GVBD with increasing mean oocyte diameter has been demonstrated in several species (Daniel *et al.,* 1989; Eppig and Schroeder, 1989; Hirao *et al.,* 1994; Bao *et al.,* 2000) although few data are available for humans.

The findings of Chapter 7 provide data on the growth of human oocytes during IVM from two sources, unstimulated women with PCO and those undergoing ICSI treatment. Similar to the findings of Durinzi *et al.* (1995) a threshold of ~ 103μm for maturation in vitro for oocytes obtained from patients with PCO was observed, however in contrast to data obtained by Durinzi *et al.* (1995), oocytes were observed to reach meiotic maturity in vitro with an oocyte diameter considerably less than

115μm. Furthermore, none of the oocytes from stimulated patients undergoing ICSI and having initial diameters of ≥115μm matured in vitro. Measurements of oocyte diameter indicated that detectable oocyte growth continues in vitro, which is a new observation not previously reported, and maturation of oocytes on both the day of oocyte recovery and the day of insemination of mature oocytes was related to oocyte diameter. Oocyte diameter (within the oolemma) proved the most useful and reliable measure, superior to measurement of total oocyte + zona diameter, zona pellucida thickness and PVS, in terms of its relationship with subsequent oocyte behaviour in vitro. A response to FF-MAS in terms of oocyte growth was evident in immature oocytes from both sources of patients. Mature, immature and atretic oocytes from patients with PCO cultured in FF-MAS (10 or 30μg/ml) became significantly different in vitro in terms of oocyte diameter. This was not true of oocytes cultured in control conditions, or those obtained from patients undergoing ICSI treatment. However, significant growth of oocytes obtained from patients undergoing ICSI was indicated by enlargement of oocyte diameter between the day of oocyte recovery and the day of insemination when oocytes were cultured in the presence of FF-MAS.

These data therefore suggest that FF-MAS may have a positive effect upon oocyte size during maturation culture, which may be related to its activity in promoting maturation and possibly developmental competence, as demonstrated in Chapter 5. This applied to oocytes without cumulus, or with a limited amount, which did not preclude visualization of the oolemma, and therefore raises questions about the control of oocyte growth, which is generally believed to be controlled substantially by cumulus-oocyte interaction. The mechanism of such an effect by FF-MAS is uncertain and its elucidation would require further experimentation.

The work conducted has resulted in a number of original observations. The potential of FF-MAS as a natural stimulator of oocyte maturation has been confirmed in humans, as anticipated from previous studies in mice, and the ongoing growth of human oocytes associated with further development during maturation culture has been presented and quantified for the first time. The potential of FF-MAS to affect the growth of oocytes directly, in both cumulus-enclosed and cumulus free conditions in vitro is also a novel finding. However, a number of outstanding questions have arisen

from this work, which could be addressed further in future studies. For example, the mechanism of action of FF-MAS remains uncertain and hence the molecular signals, which may influence the oocyte, require elucidation. FF-MAS appeared not to have any discernable effect, within my small cohort of oocytes, upon the structure or organization of chromosomes and spindles. However, the lack of normal material for comparison requires future study of freshly collected mature oocytes. It would also be interesting to test the effects of other factors promoting oocyte maturation, notably FSH, upon the non-invasive measure of oocyte diameter to assess whether diameter per se is a useful marker of developmental potential.

Finally, this series of experiments has provided insights into the techniques and culture conditions necessary to achieve IVM and to promote developmental competence in human embryos. They form a foundation for a future clinical trial of IVM and have contributed to our advancing knowledge on the future prospects of this promising treatment.

Chapter 9

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Appendix I

Figure A1 Meiosis and follicular growth in the female

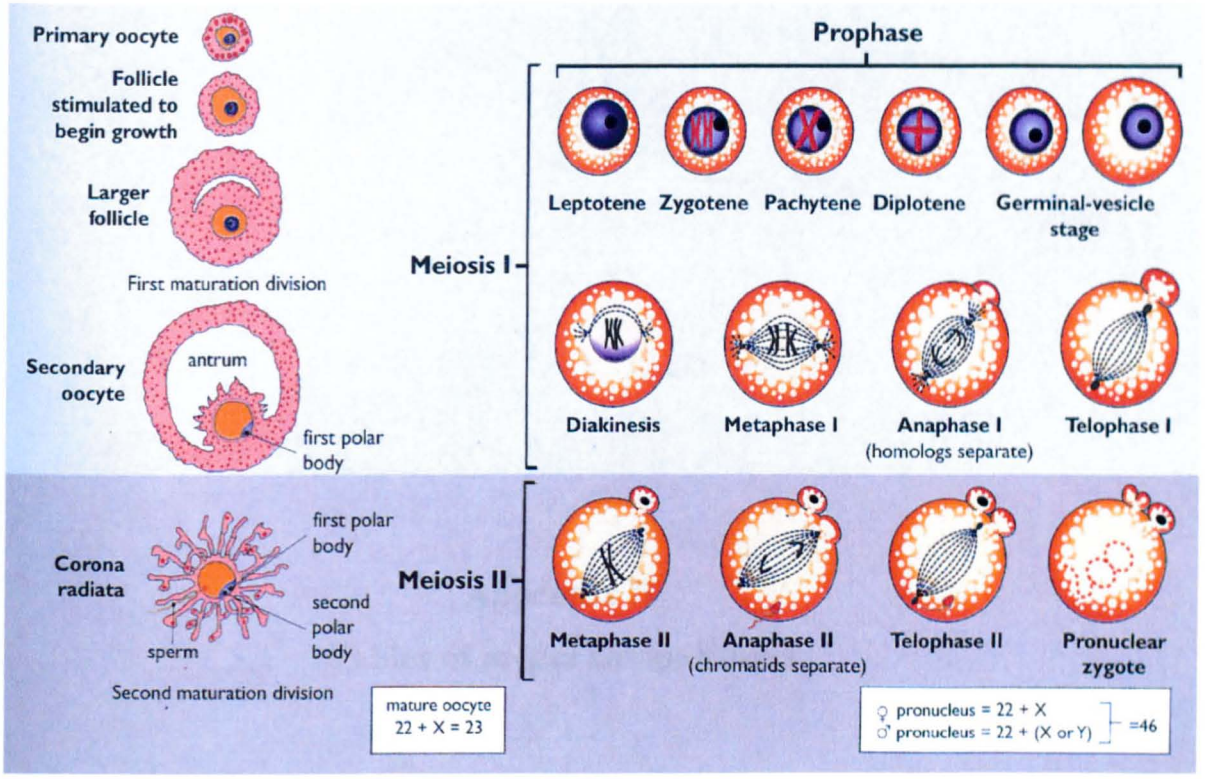

Figure A1 Meiosis and follicular growth in the female

(Taken from Veeck, 1999)

Appendix II

Tables of media compositions

Appendix II

Tables of media compositions

Table A1 Composition of EBSS 10× (Gibco)

Component	g/L
Inorganic salts:	
$CaCl_2$ (anhyd.)	2.00
KCl	4.00
$MgSO_4 \cdot 7H_2O$	2.00
NaCl	68.00
$NaH_2PO4 \cdot H_2O$	1.40
Other components:	
D-Glucose	10.00
Phenol Red	0.10

Table A2 Composition of PBS (Gibco)

Component	g/L
Inorganic salts:	
$CaCl \cdot 2H_2O$	0.132
KCl	0.20
KH_2PO4	0.20
$MgCl_2 \cdot 6H_2O$	0.10
NaCl	8.00
Na_2 HPO4	1.15

Table A3 Composition of Hams F10 (ICN Biomedicals)

Component	mg/L
Inorganic salts:	
$CaCl_2 2H_2O$	44.00
$CuSO_4$	0.0016
$FeSO_4 \cdot 7H_2O$	0.834
$MgSO_4$	74.60
KCl	285.00
KH_2PO_4	83.00
NaCl	7400.00
Na_2HPO_4	156.20
$ZnSO_4 \cdot 7H_2O$	0.0288
Other components:	
Dextrose	1100.00

HEPES	4770.00
Hypoxanthine	4.08
Lipoic Acid	0.206
Phenol Red Sodium Salt	1.240
Sodium Pyruvate	110.00
Thymidine	0.727
Amino acids:	
L-Alanine	8.910
L-Arginine·HCl	210.700
L-Asparagine·H_2O	15.010
L-Aspartic Acid	13.310
L-Cysteine HCl·H_2O	35.130
L-Glutamic Acid	14.710
Glycine	7.510
L-Histidine HCl·H_2O	20.960
L-Isoleucine	2.620
L-leucine	13.120
L-Lysine·HCl	29.300
L-Methionine	4.480
L-Phenylalanine	4.960
L-Proline	11.510
L-Serine	10.510
L-Threonine	3.570
L-Tryptophan	0.610
L-Tyrosine	1.810
L-Valine	3.510
Vitamins:	
Biotin	0.024
Choline Chloride	0.698
D-Calcium Pantothenate	0.715
Folic acid	1.320
Myo-Inositol	0.541
Nicotinamide	0.611
Pyridoxine HCl	0.206
Riboflavin	0.376
Thiamine HCl	1.012
Vitamin B12	1.360

Table A4 Composition of M199 2x (Gibco)

Component	mg/L
Inorganic salts:	
$CaCl_2 \cdot 2H_2O$	528.00
$Fe(NO_3)_3 \cdot 9H_2O$	1.40
KCl	800.00
$MgSO_4 \cdot 7H_2O$	400.00
NaCl	13600.00
$NaHCO_3$	2500.00
$NaH_2PO4 \cdot 2H_2O$	316.00
Other components:	
Adenine Sulphate	20.00
Adenosine-5-triphosphate	2.00
Adenosine-5-phosphate	0.40
Cholesterol	0.40
2-Deoxy-D-ribose	1.00
D-Glucose	2000.00
Glutathione (reduced)	0.10
Guanine	0.60
Hypoxanthine	0.60
Ribose	1.00
Sodium Acetate $\cdot 3H_2O$	166.00
Thymine	0.60
Tween 80	40.00
Uracil	0.60
Xanthine	0.60
Amino acids:	
DL-Alanine	100.00
L-Arginine $\cdot$ HCl	140.00
DL-Aspartic Acid	120.00
L-Cysteine HCl	0.22
L-Cysteine	40.00
DL-Glutamic Acid	267.32
L-Glutamine	200.00
Glycine	100.00
L-Histidine HCl $\cdot H_2O$	43.76
L-Hydroxyproline	20.00
L-Isoleucine	80.00
DL-leucine	240.00
L-Lysine $\cdot$ HCl	140.00
DL-Methionine	60.00
DL-Phenylalanine	100.00
L-Proline	80.00
DL-Serine	100.00

DL-Threonine	120.00
L-Tryptophan	20.00
L-Tyrosine	80.00
DL-Valine	100.00
Vitamins:	
Ascorbic acid	0.10
D-Biotin	0.02
Calciferol	0.20
D-Calcium Pantothenate	0.02
Choline Chloride	1.00
Folic acid	0.02
i-Inositol	0.10
Menadione	0.02
Nicotinic acid	0.05
Nicotinamide	0.05
Para-aminobenzoic acid	0.10
Pyridoxal HCl	0.05
Pyridoxine HCl	0.05
Riboflavin	0.02
Thiamine HCl	0.02
Vitamin A (acetate)	0.20

Table A5 Composition of P1 medium with gentamycin (Irvine Scientific)

Component	**mM**
NaCl	101.6
KCl	4.69
$MgSO_4$	0.20
$CaCl_2$-$2H_2O$	2.04
$NaHCO_3$	25.0
Na Pyruvate	0.33
Na Lactate	21.4
Taurine	0.05
Na Citrate	0.15mg/L
Phenol Red	0.005g/L
Gentamicin	10µg/ml

Table A6 Composition of S1 and S2 media (Scandinavian IVF Science)

S1 Medium	S2 Medium	
NaCl	NaCl	EDTA
KCl	KCl	Alanine
KH_2PO4	KH_2PO4	Asparagine
$MgSO_4$	$CaCl{\cdot}2H_2O$	Aspartic acid
hSA	$MgSO_4$	Glutamine
$NaHCO_3$	$NaHCO_3$	Glycine
Na Pyruvate	Na Pyruvate	Proline
Na Lactate	Na Lactate	Serine
$CaCl{\cdot}2H_2O$	Taurine	Calcium Pantothenate
Glucose	Glucose	Choline Chloride
EDTA	Threonine	Folic acid
Alanine	Estradiol	i-Inositol
Asparagine	Progesterone	Nicotinamide
Aspartic acid	Arginine	Pyridoxine
Glutamine	Cystine	Riboflavin
Glycine	Histidine	Thiamine
Proline	Isoleucine	Insulin
Serine	Lysine	hSA
	Methionine	

Table A7 Composition of Gardner's G1.2 and G2.2 sequential media (Scandinavian IVF Science)

Component		G1.2 medium	G2.2 medium
NaCl	(mM)	85.16	85.16
KCl	(mM)	5.5	5.5
$NaH_2PO_42H_20$	(mM)	0.5	0.5
$CaCl_22H_20$	(mM)	1.8	1.8
$MgSO_4.7H_20$	(mM)	1.0	1.0
$NaHCO_3$	(mM)	25.0	25.0
Na lactate (60% syrup)	(mM)	21.0 (10.5 L-isomer)	11.74 (5.87 L-isomer)
Na pyruvate	(mM)	0.32	0.10
Glucose	(mM)	0.50	3.15
Glutamine	(mM)	1.0	1.0
Taurine	(mM)	0.1	0.0
Non-essential amino-acids		All	All
Essential amino-acids		None	All
Ethylenediaminetetraacetic acid	(mM)	0.1	0
BSA	(g/l)	2.0	2.0
Penicillin	(g/l)	0.06	0.06
Streptomycin	(g/l)	0.05	0.05
Phenol red	(g/l)	0.01	0.01

Adapted from Barnes *et al.* (1995)

Appendix III

Figure A2 Representative images of oocytes demonstrating the variable extent of cumulus cover

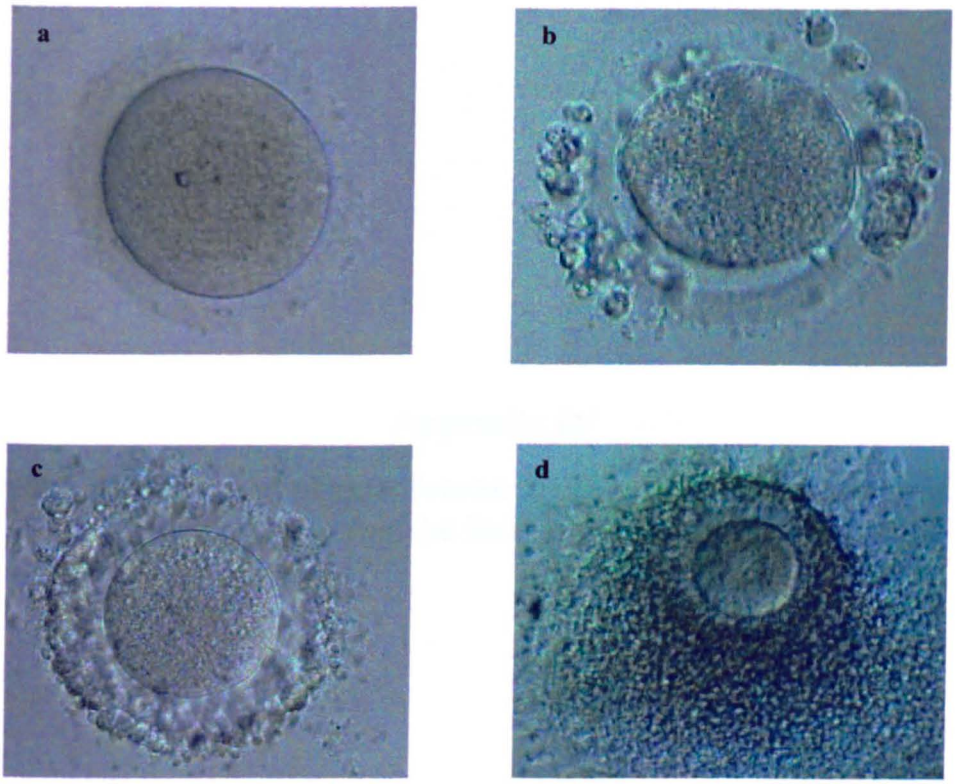

Figure A2 Representative images of oocytes demonstrating the variable extent of cumulus cover. Cumulus was graded subjectively from 0-3.

a) Grade 0- Oocyte devoid of cumulus
b) Grade 1- Partially covered zona
c) Grade 2- Completely covered zona
d) Grade 3- Substantial multilayered cumulus cover

Appendix IV

Figure A3 Image of oocyte demonstrating the various parameters measured using the image analysis package

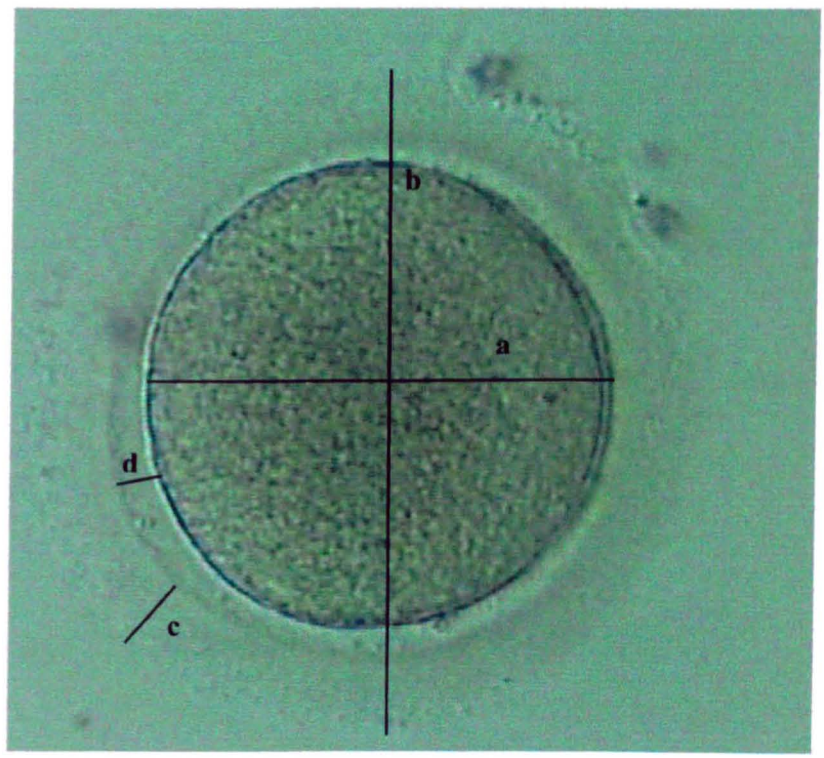

Figure A3 Image of oocyte demonstrating the various parameters measured using the image analysis package.

a. Mean diameter of oocyte- final measurement was calculated by measuring the mean length of diameters as far as the oolemma at two-degree intervals and passing through the object's centroid.
b. Mean diameter of oocyte + zona- final measurement was calculated as for oocyte diameter, but extending the diameter measurement to the outer surface of the zona pellucida.
c. Mean zona pellucida thickness- final measurement was calculated after measuring the zona pellucida thickness at 2μm intervals.
d. Perivitelline space (PVS)- final measurement was generated by calculating the minimum and the maximum distance between the inner zona and the oolemma and calculating a mean PVS from this.

Appendix V

Publication-Cavilla *et al.* (2001)

Human Reproduction Vol.16, No.3 pp. 547–555, 2001

The effects of meiosis activating sterol on in-vitro maturation and fertilization of human oocytes from stimulated and unstimulated ovaries*

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The object of this study was to assess functional maturation *in vitro* by obtaining data on the fertilization and embryonic competence of human oocytes with or without exposure to meiosis activating sterol (MAS) during maturation *in vitro*. Immature oocytes were either collected from unstimulated patients with polycystic ovaries (PCO) during gynaecological surgery, or were donated by patients undergoing a cycle of intracytoplasmic sperm injection (ICSI) treatment including ovarian stimulation with gonadotrophins. PCO oocytes had variable cumulus cover, which was retained during culture while those from ICSI patients were cultured without cumulus. The study included 119 oocytes from PCO patients and 72 from ICSI patients. The oocytes were allowed to mature *in vitro* for up to 46 h in the presence or absence of MAS. Mature oocytes were inseminated by ICSI with fertile donor spermatozoa and embryo development was monitored *in vitro*. MAS (30 μg/ml) significantly increased the survival of oocytes from PCO patients ($P < 0.01$) but did not significantly affect the proportion completing maturation *in vitro*. For the ICSI patients, >90% of oocytes survived in all culture groups, regardless of MAS addition, however MAS (10 or 30 μg/ml) significantly increased the proportion of oocytes maturing *in vitro* ($P < 0.05$). The apparent tendency towards improved subsequent development *in vitro* will require larger numbers of oocytes for evaluation. Oocytes from ICSI patients matured more rapidly *in vitro* than those from PCO patients. Our results show positive effects of MAS on human oocytes, confirming previous data in mice. This work may have implications for the future clinical application of IVM.

Key words: human/ICSI/in-vitro maturation/meiosis activating sterol/oocyte

Introduction

The final 36 h of human oocyte formation are critical for the normal functioning of the resulting gamete. During this time, oocytes resume meiosis from their prenatal arrest in diplotene and progress to metaphase II (MII). They also undergo 'cytoplasmic maturation', a poorly defined process, in preparation for fertilization and early embryo development, which largely depends upon oocyte constituents until after embryonic genome activation. Prior readiness of the oocyte to progress through the final maturation phase and attain its full developmental potential relies upon an appropriate follicular environment and the type of follicle from which the oocyte originates is crucial for its subsequent function. It has been known for many years that immature human oocytes removed from large follicles will mature spontaneously (Edwards, 1965a,b); however, the developmental competence of in-vitro matured (IVM) human oocytes is low (Veeck *et al.*, 1983; Barnes *et al.*, 1996; Coskun *et al.*, 1998), probably due to disruption of the normal follicular control mechanisms regulating this important stage of development. For this reason, in-vivo maturation has been preferred for oocyte preparation for IVF, despite the need for large doses of exogenous hormones, with their attendant risks and costs (Russell, 1999). This is in contrast to some animal species where IVM is commonly used to obtain viable oocytes for research or commercial purposes (Trounson *et al.*, 1996).

*Presented in part at the British Fertility Society Conference, Sheffield, UK, 15–17 April 1998, and at the 15th meeting of the European Society of Human Reproduction and Embryology, Tours, France, 27–30 June 1999.

Immature oocyte retrieval combined with IVM could offer an alternative to the current ovarian stimulation protocols used in IVF, however, efficient maturation, fertilization and embryo development are needed before IVM can be applied routinely in the treatment of human infertility. Recently, several groups have reported improved viability of human IVM oocytes using various protocols including partial, minimal or no exogenous hormonal stimulation (Wynn, *et al.*, 1998; Chian *et al.*, 1999; Jaroudi *et al.*, 1999). The content of the medium used for IVM may also affect the outcome. A key experiment in the 1990s, which re-awakened interest in human IVM, used human follicular fluid to supplement the culture medium (Cha *et al.*, 1991) but, with the growing preference for defined media, the roles of individual factors are now being assessed and the inclusion of biological fluids is declining.

Various hormones included in the culture medium have been reported to promote oocyte maturation and subsequent embryo development, e.g. epidermal growth factor (EGF; Gómez *et al.*, 1993a,b; Goud *et al.*, 1998) or FSH (Barnes *et al.*, 1996; Durinzi *et al.*, 1997) with or without human chorionic gonadotrophin (HCG; Jaroudi *et al.*, 1997; Liu *et al.*, 1997). In addition, intracytoplasmic sperm injection (ICSI) of IVM oocytes has increased the likelihood of normal fertilization (Barnes *et al.*, 1996; Cha and Chian, 1998).

Meiosis activating sterols (MAS) have been purified from human follicular fluid (FF-MAS: 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol) and bull testicular tissue (T-MAS: 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol) (Baltsen and Byskov, 1999). FF-MAS is present in human pre-ovulatory follicular fluid at concentrations of ~1.3 μmol/l, and is an intermediary occurring naturally in the biosynthetic pathway between lanosterol and cholesterol (Byskov *et al.*, 1995). It activates meiotic resumption of both cumulus-enclosed and denuded mouse oocytes *in vitro* (Byskov *et al.*, 1995, 1999; Hegele-Hartung *et al.*, 1999). MAS is synthesized by cumulus cells of intact oocyte–cumulus–complexes in response to FSH stimulation (Byskov *et al.*, 1998) and the concentrations of MAS in pre-ovulatory follicular fluid samples are correlated with the ability of the associated oocyte to fertilize and cleave (Byskov *et al.*, 1998). A histological study of 81 human oocytes suggested that the completion of maturation *in vitro* might be promoted by FF-MAS (Grøndahl *et al.*, 2000). Our focus has been to assess functional maturation by obtaining data on the fertilization and embryonic competence of human oocytes after FF-MAS exposure during IVM. No data on this subject have been published previously.

The prevalence of polycystic ovaries (PCO) in 'normal' adult women is ~20% (Polson *et al.*, 1988; Clayton *et al.*, 1992). PCO may be associated with infertility and such patients may be hyper-responsive to the gonadotrophin stimulation normally used for IVF. A classical polycystic ovary has several antral follicles up to ~10 mm diameter around the periphery of the ovary. Although these follicles are not actively growing, they contain immature oocytes which may be capable of further development (Trounson *et al.*, 1994; Chian *et al.*, 1999). Women with PCO therefore represent a patient group most likely to benefit from IVM.

There may be additional potential benefits from IVM, for example, allowing rapid oocyte collection in women with cancer who cannot spend time undergoing a normal IVF cycle before sterilizing chemotherapy. The potential of an embryo largely reflects the competence of the oocyte and it might become possible, in future, to select immature oocytes with the greatest potential before fertilization, avoiding some of the ethical dilemmas of embryo selection.

These factors, together with the developing knowledge on mechanisms controlling follicle and oocyte growth and early embryology suggest that the time is right for IVM to be applied with caution in patient treatment. We have performed this preclinical research study to assess the effects of meiosis activating sterol (FF-MAS), a naturally occurring stimulator of meiosis, upon the maturation and preimplantation development of immature human oocytes from two different sources: (i) unstimulated patients with PCO and (ii) patients undergoing a fully stimulated cycle of ICSI treatment.

Table I. Clinical details of patients with polycystic ovaries (PCO) who donated immature oocytes

Patients (*n*)	17
Patient age (years)[a]	28.1 ± 3.9 (22–35)
Menstruation	
Regular (28–35 days)	3
Oligomenorrhoeic	10
Amenorrhoeic (2°)	2
Data not recorded	2
No of oocytes collected[a]	
Live	7.0 ± 4.1 (1–19)
Atretic	4.9 ± 3.7 (0–14)

[a]Values are presented as mean ± SD, with range in parentheses.

Table II. Clinical details of patients undergoing intracytoplasmic sperm injection (ICSI) who donated immature oocytes

Patients (*n*)	28
Patient age (years)[d]	32.4 ± 3.4 (27–40)
No. of previous ICSI attempts[d]	0.8 ± 0.9 (0–3)
Total no. of oocytes collected[d]	16.3 ± 6.2 (5–27)
Fertilization rate (%) (2PN)[e]	71.5
Ongoing pregnancy rate (%)	10/27[a,b] (37)
Pregnancy losses	1[c]
No. of cleaving embryos frozen	2.6 ± 2.5
No. of 2PN frozen	1.5 ± 1.7
No. of immature oocytes donated to study	2.6 ± 0.9 (1–6)

[a]For one cycle, all embryos frozen.
[b]One set of twins.
[c]Biochemical pregnancy.
[d]Data are mean ± SD, with range in parentheses.
[e]Oocytes with two pronuclei (2PN)/oocytes injected.

Materials and methods

Oocytes from two groups of patients were studied: (i) those with unstimulated PCO and (ii) those undergoing ICSI treatment, which included ovarian stimulation according to a standard 'long' down-regulation protocol. Information on the patient groups is presented in Tables I and II. This study was approved by Coventry Research Ethics Committee and licensed by the Human Fertilisation and Embryology Authority.

Unstimulated PCO patients

These patients were attending gynaecology or infertility clinics at the Walsgrave Hospital, Coventry. Patients having symptoms of PCO (Adams *et al.*, 1986) and who were undergoing treatment, including laparoscopy, were invited to participate in the study.

Inclusion criteria were: (i) PCO, diagnosed primarily by the ultrasound appearance of the ovaries, but additionally by a history of oligomenorrhoea or amenorrhoea, and blood hormone measurements; (ii) no drug therapy for infertility in the 4 months before surgery; and (iii) requiring diagnostic laparoscopy and/or laser drilling of the ovaries. Patients wishing to participate provided written consent. Laparoscopy was scheduled irrespective of the stage of the menstrual cycle.

Retrieval of immature oocytes

Oocyte retrieval procedures were carried out in the afternoon in theatres on the same site but separate from the main embryology laboratory. A previously described technique (Trounson *et al.*, 1994) was followed using a purpose-designed 17 gauge needle (Cook IVF, Letchworth, UK) and a laparoscopic approach. All visible follicles were punctured and aspirated using 80–100 mmHg suction. Measurement of follicular diameter was not possible by direct laparoscopic inspection since the follicles were relatively small (<10 mm) and the stroma was dense. The fluid was collected into 10 ml sterile plastic tubes (Fahrenheit Laboratory Supplies, Milton Keynes, UK) containing 2 ml pre-warmed, heparinized (3 IU/ml; Leo Laboratories Ltd, Buckinghamshire, UK) Ham's F10 medium (ICN Pharmaceuticals Ltd, Thame, UK) buffered with 20 mmol/l HEPES and supplemented with 0.5% human serum albumin (HSA; Immuno Ltd, Dunton Green, UK). The aspirates were maintained at 37°C in a thermostatic hot-block (Grant, Cambridge, UK) and transported to the embryology laboratory in a portable incubator (Cell Trans 4016 Transport Incubator; Labotech). The oocyte aspiration procedure took ~40 min and the journey <10 min.

Follicular aspirates were placed into an Em-Con filter of 75 μm pore size (Immuno Systems, Spring Valley, WI, USA) which had been pre-rinsed with warmed heparinized Ham's F10 medium as above. The aspirates were flushed with 250 ml of the same medium to remove contaminating blood cells and the filter retentate was transferred to sterile dishes (Falcon, Fahrenheit).

Oocyte–cumulus masses and free oocytes were identified using a dissecting microscope (Leica Microsystems, Milton Keynes, UK), equipped with a heated stage, and transferred to tissue culture dishes (Nunclon; Gibco, Life Technologies, Paisley, UK) containing 2 ml IVM medium. IVM medium comprised Medium 199 (Gibco) supplemented with 0.23 mmol/l sodium pyruvate (Sigma, Poole, UK), 50 IU/ml penicillin G and 50 μg/ml streptomycin (Gibco). The IVM medium was prepared on the morning of oocyte retrieval and dishes of medium were pre-equilibrated in a humidified incubator at 37°C, containing 5% CO_2 in air. The preparation of MAS-containing IVM medium is explained below.

The level of cumulus cover surrounding the oocyte was graded subjectively from 0–3, where 0 = devoid of cumulus; 1 = partially covered zona; 2 = completely covered zona; and 3 = substantial multi-layered cumulus cover. Individual viable oocytes were randomly allocated to one of three concentrations of MAS using tables of random numbers.

An oocyte was considered to be viable if it had an intact oolemma, a light coloured cytoplasm and a regular-appearing spherical shape. In previous experiments using the fluorescent vital stains, carboxyfluorescein diacetate (CFDA) and propidium iodide (PI), these features had been confirmed as identifying viable oocytes. Where possible, it was recorded whether a germinal vesicle (GV) was present in the ooplasm, and any other features of oocyte morphology. Oocytes obscured by tightly surrounding cumulus cells (cumulus enclosed; CE) were presumed to contain a GV. The initial assessment of maturity was performed ~2 h after oocyte retrieval.

Images of oocytes were collected daily via a video link on an inverted high power microscope (Olympus IX 70) employing Hoffman optics and Image Pro-plus software. Oocytes which were clearly atretic on collection, e.g. dark cytoplasm and irregular shape, were noted before discarding.

ICSI patients

Immature oocytes were donated by women undergoing ICSI at the Centre for Reproductive Medicine, Walsgrave Hospital. Women wishing to participate gave written consent prior to the oocyte retrieval. Patient preparation, oocyte retrieval and embryological procedures were performed using the centre's standard protocols (Garello *et al.*, 1999). Any immature oocytes identified when oocytes were stripped of cumulus in preparation for ICSI were randomly allocated to MAS treatment groups using random number tables; thereafter, they were treated identically to oocytes from PCO patients. Oocytes were considered immature if they contained a GV or had undergone germinal vesicle breakdown (GVBD) but had not released a polar body.

Meiosis activating sterol (FF-MAS)

FF-MAS was purified from human follicular fluid as described (Byskov *et al.*, 1995; Baltsen and Byskov, 1999). MAS was stored at −20°C in *n*-heptane under N_2 in glass vials. Before use, a stream of nitrogen gas was used to evaporate the heptane from a known quantity of MAS which was dissolved in a known small volume of absolute ethanol (EtOH) and added to the prepared IVM media in glass tubes to final concentrations of 10 and 30 μg/ml (24.4 and 73.2 μM). EtOH alone, treated in the same manner, was added to controls. All wells contained a final concentration of 0.5% EtOH. MAS/EtOH was added to the cultures ~1 h before oocyte retrieval.

Volumes of 100 μl IVM medium containing the various concentrations of MAS were set up in a sterile 96-well plate (Nunclon) which also contained medium in the surrounding wells to increase humidity and reduce evaporation. Cultures were housed in a humidified incubator (37°C, 5% CO_2 in air). An oil overlay was not used, thus avoiding oil phase extraction of MAS. Control experiments demonstrated that the osmolarity of cultures maintained under these conditions varied by <1% after 24 h.

Up to four oocytes were co-incubated in each well. The oocytes were examined after 23–24 h and their maturational stage recorded. Oocytes which had extruded a polar body were considered mature and removed for ICSI. 40–48 h after initiation of culture, any remaining cumulus cells were removed by pipetting and all oocytes were assessed microscopically. Mature oocytes were injected with known fertile donor spermatozoa according to the Centre's established ICSI protocol (Garello *et al.*, 1999). Frozen stored spermatozoa from two fertile donors were used for the entire study.

After injection, oocytes were transferred individually to S1 or G1 medium (Scandinavian IVF Science, Sweden) in 100 μl drops under oil (Ovoil; Scandinavian IVF Science). Dishes were pre-equilibrated at 37°C in an atmosphere of 5% CO_2 in air for 1 day before use. The oocytes were checked for fertilization 14–18 h post-injection using standard procedures of pronuclear assessment. Zygotes and cleaving embryos were observed and moved to fresh drops daily. Computerized photographic images were collected each day until the embryo arrested or degenerated.

Statistical analyses

The proportions of oocytes in groups exposed to different concentrations of MAS were compared using $p \times q$ χ^2 test (Campbell, 1989).

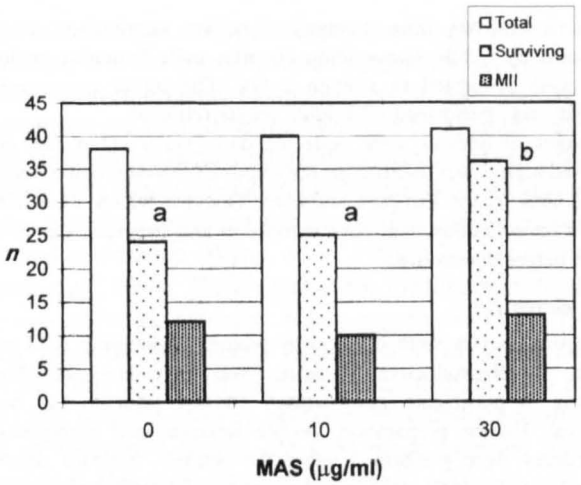

Figure 1. In-vitro maturation of immature oocytes (n = 119) collected from patients (n = 17) with polycystic ovaries and cultured with or without meiosis activating sterol (MAS). Bars with different letters are significantly different ($P < 0.01$).

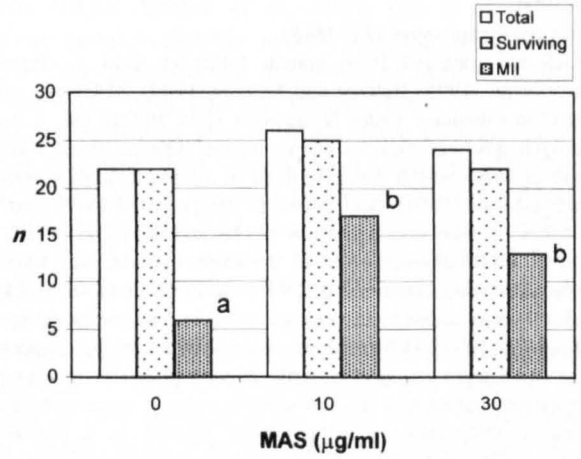

Figure 2. In-vitro maturation (IVM) of immature oocytes (n = 72) donated by patients undergoing intracytoplasmic sperm injection (ICSI) (n = 28) and cultured with or without meiosis activating sterol (MAS). Bars with different letters are significantly different ($P < 0.05$).

Scatter plots were assessed for correlation using regression analysis. $P < 0.05$ was considered to be statistically significant. Student's t-test was used to compare the numbers of viable oocytes in the two patient groups.

Results

A total of 119 viable immature oocytes were collected from 17 PCO patients with unstimulated ovaries and 72 immature oocytes were donated by 28 ICSI patients. Clinical details of the patient groups are presented in Tables I and II.

Figure 1 shows the results of MAS exposure of oocytes obtained from PCO patients. MAS at a concentration of 30 μg/ml significantly increased oocyte survival ($P < 0.01$, p × q χ^2 test, Campbell, 1989); 90% survival in 30 μg/ml compared with 62 and 63% for 10 μg/ml and control respectively. The increased survival of oocytes in 30 μg/ml MAS was not associated with an alteration in the proportion of surviving oocytes which matured, which remained at ~45%.

Figure 2 shows the results of MAS exposure of immature oocytes from ICSI patients. Oocyte survival was >90% in all groups, regardless of MAS concentration. However, in this patient group, the presence of MAS at 10 or 30 μg/ml significantly increased the proportion of oocytes maturing ($P < 0.05$).

The proportions of maturing oocytes fertilized by ICSI and subsequently cleaving in either PCO or ICSI groups are presented in Tables III and IV, but the apparent differences were not tested statistically in view of the low numbers of oocytes reaching this stage.

Figure 3 shows that significantly more immature oocytes per patient were obtained from PCO patients in comparison

Table III. Fertilization rates (no. of two pronuclear oocytes/oocytes surviving ICSI) for in-vitro matured oocytes arising from polycystic ovaries (PCO) or intracytoplasmic sperm injection (ICSI) patients. Values in parentheses are percentages

	Meiosis activating sterol (μg/ml)		
	0	10	30
PCO	4/10 (40)	2/9 (22)	7/11 (64)
ICSI	2/5 (40)	6/11 (55)	6/10 (60)

Table IV. Cleavage rates (no. of cleaving embryos/two pronuclear oocytes) for in-vitro matured oocytes arising from polycystic ovaries (PCO) or intracytoplasmic sperm injection (ICSI) patients

	Meiosis activating sterol (μg/ml)		
	0	10	30
PCO	2/4	2/2	5/7
ICSI	0/2	3/6	5/6

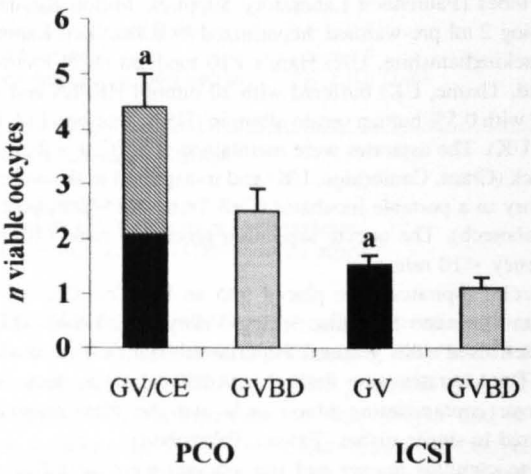

Figure 3. Maturity at collection of oocytes recovered from polycystic ovaries (PCO) and intracytoplasmic sperm injection (ICSI) patients. GV = germinal vesicle; GVBD = germinal vesicle breakdown; CE = cumulus enclosed (GV assumed, but not visible). [a]Significantly different ($P < 0.01$).

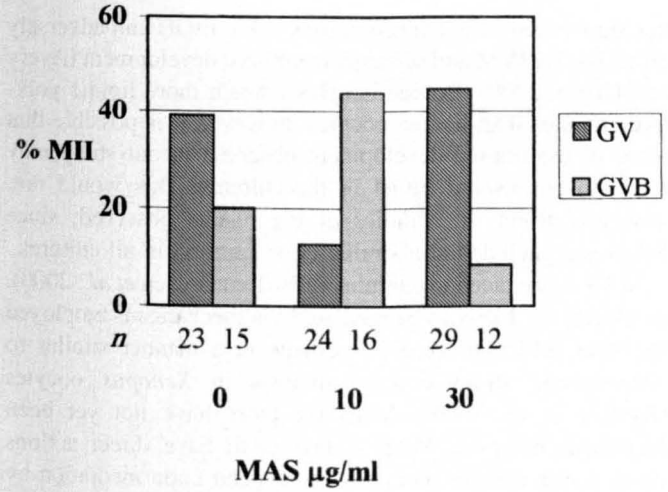

Figure 4. Maturation of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) oocytes from patients with polycystic ovaries (PCO) in the presence or absence of meiosis activating sterol (MAS). n = number of oocytes in each group.

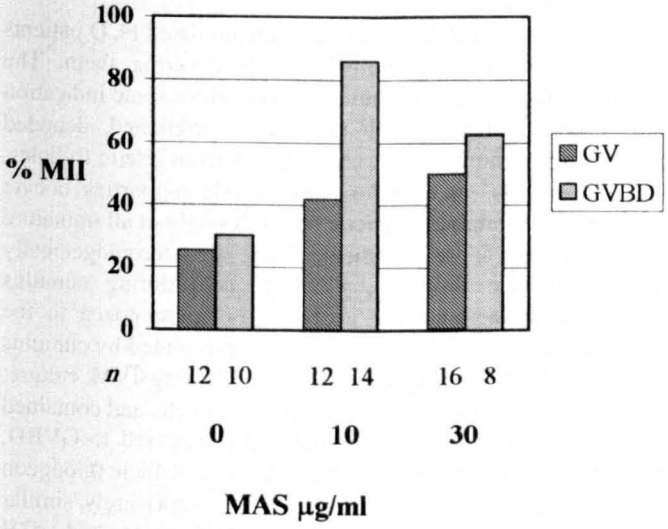

Figure 5. Maturation of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) oocytes from patients undergoing intracytoplasmic sperm injection (ICSI) in the presence or absence of meiosis activating sterol (MAS). n = number of oocytes in each group.

with ICSI patients ($P < 0.01$), but surprisingly, there was no significant difference in the proportion of oocytes that had undergone GVBD between the PCO and ICSI patient groups.

Figures 4 and 5 show the maturation *in vitro* to MII of oocytes collected at the GV and GVBD stages for both patient groups. In PCO patients (Figure 4), the proportion of oocytes in both categories reaching MII was highly variable. Cultured immature oocytes from ICSI patients had a low maturation rate (either GV or GVBD at collection) but this was improved by addition of MAS, as shown in Figure 5. For the oocytes that failed to mature in the ICSI group, approximately equal numbers arrested at or after the GV stage.

Figure 6 shows the rates of atresia occurring *in vitro* after collection of apparently viable oocytes. Especially high levels of atresia were noted in GVBD oocytes from PCO patients. Overall the PCO oocytes appeared more susceptible than ICSI

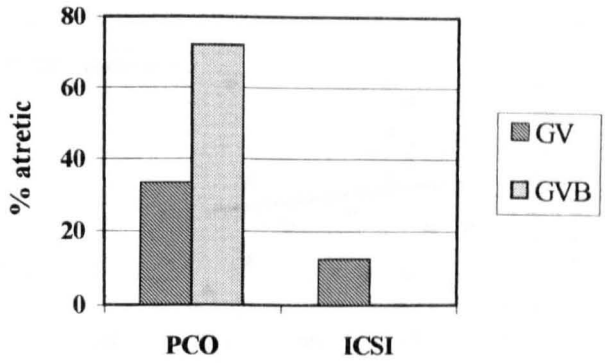

Figure 6. Proportions of germinal vesicle (GV) and germinal vesicle breakdown (GVBD) oocytes becoming atretic *in vitro* after collection from polycystic ovaries (PCO) or intracytoplasmic sperm injection (ICSI) patients.

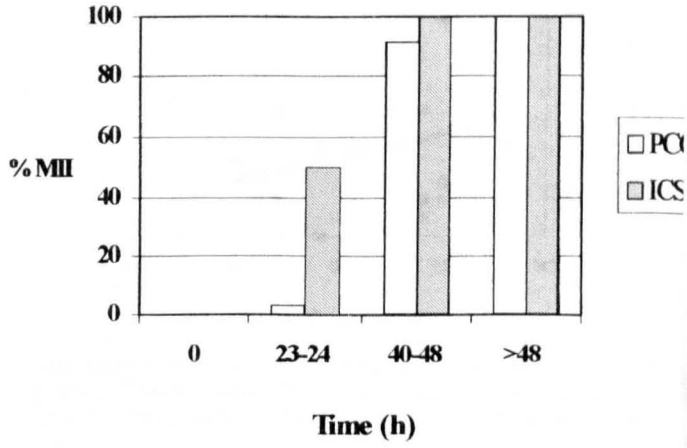

Figure 7. Cumulative time course of maturation *in vitro* for oocytes from polycystic ovaries (PCO) and intracytoplasmic sperm injection (ICSI) patients.

oocytes. In the ICSI group, atresia was confined to oocytes collected at the GV stage.

The time course of oocyte maturation was different in the PCO and ICSI groups. Of those oocytes maturing to MII *in vitro*, 50% in the ICSI group had reached MII by 23–24 h compared with <5% for PCO patients (Figure 7). The majority (89%) of PCO oocytes that matured to MII, did so on the second day of IVM culture.

There was no significant relationship between the numbers of viable or atretic oocytes collected and patient age in PCO patients ($r = 0.141$ and 0.268 respectively), as shown in Figure 8. Similarly, there was no relationship between the number of viable oocytes and the number of days since the last menstrual period ($r = 0.040$) or patient weight ($r = 0.264$) in the PCO patients (data not shown).

Discussion

Our results demonstrate, for the first time, the significant effects of MAS on in-vitro survival and maturation of human oocytes. Immature oocytes collected from two different patient groups, unstimulated PCO patients and stimulated ICSI patients, have been shown to differ in terms of oocyte function *in vitro*, including the proportions maturing and the speed of

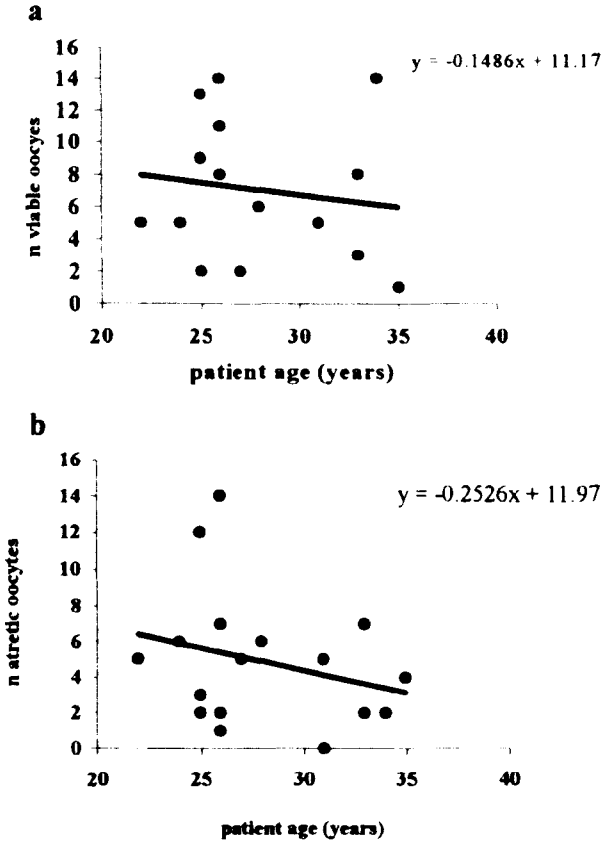

Figure 8. Scatterplot of the number of viable (**a**) and atretic (**b**) oocytes retrieved with age of polycystic ovaries (PCO) patient. No significant correlations were present (**a**) $r = 0.141$; (**b**) $r = 0.268$.

maturation *in vitro*. These differences may reflect patient-related differences in the origin of the oocytes, including the stage of follicle development at oocyte retrieval, the endocrine status or atresia of the follicles and the presence or absence of supporting cumulus cells. These results will be important for the future application of IVM as a potential treatment in various clinical situations.

MAS significantly ($P < 0.01$) improved the survival of immature oocytes collected from unstimulated women with PCO. This is in contrast with the results obtained culturing immature oocytes from stimulated ICSI patients in whom FF-MAS did not affect oocyte survival (which was >90% in this patient group), but maturation *in vitro* was significantly increased ($P < 0.05$). The variations in embryo development in both patient groups with MAS supplementation of IVM media are also interesting, particularly in view of the compromised developmental competence which is widely believed to be associated with immature oocytes collected from PCO patients, although statistical analysis was precluded by the limited numbers of oocytes reaching the later stages in our study.

There are some difficulties of working with steroids *in vitro*. We avoided the use of oil, which would extract the steroidal MAS, but we cannot exclude the possibility of it adhering to the plastic culture vessel or being affected by albumin or other constituents in the culture medium. Similarly, the method of dissolving steroids in EtOH is widely used, but recent evidence has shown that even concentrations <1% EtOH can adversely affect bovine IVM and subsequent embryo development (Avery and Greve, 2000). Bovine oocytes contain more lipidic yolk-like granules than human oocytes; however, it is possible that some of the limited development observed in our study may relate to our use of EtOH in the cultures. This would not, however, affect the validity of the results observed, since EtOH was included at a similar concentration in all cultures.

MAS is produced in cumulus cells (Leonardsen *et al.*, 2000). Its effects are likely to be mediated via mechanisms employed by other follicular steroids, perhaps in a manner similar to progesterone stimulation of meiosis in *Xenopus* oocytes (Byskov *et al.*, 1998). MAS receptors have not yet been identified; however, MAS is known to have direct actions upon mouse oocytes and does not depend upon mediation by cumulus cells (Byskov *et al.*, 1995), unlike, e.g. gonadotrophins. The steroid environment of follicles in PCO is disturbed (e.g. Almahbobi and Trounson, 1996; Pierro *et al.*, 1997), and so the influence of MAS in this environment might differ from other circumstances.

Immature oocytes retrieved from unstimulated PCO patients may or may not have cumulus cells covering them. The presence or absence of cumulus cells provides some indication of the follicle from which the oocyte originated, denuded oocytes being more likely to have arisen from atretic follicles. The known roles of cumulus cells include supporting oocyte growth and maintaining meiotic arrest throughout all immature stages of oocyte development. They are steroidogenically active and also produce hyaluronic acid during cumulus expansion to facilitate the approach of spermatozoa to the zona pellucida at fertilization. Oocytes surrounded by cumulus cells may therefore be at an advantage during IVM culture. Most enclosed oocytes had compact corona cells and contained a GV, and many of those without had progressed to GVBD, probably as a result of incipient atresia in the follicle (Gougeon and Testart, 1986; Anderson *et al.*, 1997). Surprisingly, similar proportions of oocytes from PCO and ICSI patents had a GV or had undergone GVBD; however, these oocytes differed substantially in their predispositions towards atresia and their ability to mature to MII. The removal of cumulus cells from immature oocytes collected from ICSI cycles might have affected some aspects of their development.

The mean number of viable oocytes collected from PCO patients was 7.0, which was lower than the means of 13.8 and 13.1 obtained from anovulatory and ovulatory PCO patients respectively (Trounson *et al.*, 1994), but comparable with the average of 8.1 per patient reported in a later study (Coskun *et al.*, 1998). We did not find any specific characteristics of the PCO patients which were predictive of the numbers of oocytes retrieved; specifically, patient age, time since last menstrual period, and weight showed no significant correlations. The number of oocytes collected would be expected to reduce as the ovarian reserve declines with advancing age (Cha *et al.*, 1991; Cha and Chian, 1998; Whitacre *et al.*, 1998); however, this was not our experience in this study. One group (Barnes *et al.*, 1996) has previously reported the retrieval of a larger number of oocytes from irregularly cycling and anovulatory patients compared to regularly cycling women

(16.5 and 4.9 respectively) which relates to the relative ease of aspiration from the peripherally located antral follicles in PCO. Extremes of weight are known to affect fertility; in obese women with PCOS, fertility may be enhanced by a loss of weight. In this study, which included a range of obese and non-obese women, we observed no association between weight and oocyte yield.

Several studies have shown that oocytes aspirated after ovarian stimulation may be in various stages of maturation (Veeck *et al.*, 1983; De Vos *et al.*, 1999). Oocytes that remain immature have failed to respond to ovarian stimulation *in vivo* and may be of inherently reduced quality, they may have come from small follicles with reduced gonadotrophin sensitivity or may never have reached meiotic competence. Alternatively, they may have arisen from otherwise normal follicles which did not receive the same hormonal stimulus as others, perhaps due to their relative position in the ovary or limited blood supply. Moreover, the artificial conditions of gonadotrophin stimulation may have affected some aspects of their metabolism or development (Johnson *et al.*, 1991). Nevertheless, it has been reported that these oocytes are capable of maturing *in vitro*, fertilizing and developing normally (Cha and Chian, 1998). Our results demonstrate their responsiveness to stimulation with MAS, despite the absence of cumulus cells and their prior exposure to ovarian stimulation.

The importance of the endocrine environment in ensuring normal cytoplasmic maturation and subsequent fertilization is well known from work in animals (Moor and Trounson, 1977; Anderiesz and Trounson, 1995). The differing results obtained in this study in patients with differing endocrine profiles underline the potential for IVM success rates to be affected by the oocytes' prior exposure to physiological or pathological hormonal environments. *In vitro*, 28–30 h is required for the maturation of human oocytes to MI and 36–37 h to MII (Edwards, 1965a,b). After HCG injection to simulate a LH surge, the results are similar, the majority of the oocytes having extruded the first polar body by 36 h after HCG injection (Janssenswillen *et al.*, 1995). One group (Jamieson *et al.*, 1991) reported significantly lower IVF and cleavage rates in oocytes retrieved <36 h after the luteinizing stimulus, demonstrating the importance of appropriate timing of insemination. Previous studies have shown that immature oocytes obtained from stimulated cycles are more likely to undergo maturation than unstimulated oocytes and that the time required for their maturation is reduced (Gomez *et al.*, 1993a; Cha and Chian, 1998). Our results concur with these prior observations.

IVM can result in pregnancy (Cha *et al.*, 1991; Trounson *et al.*, 1994; Barnes *et al.*, 1995; Russell, 1998; Jaroudi *et al.*, 1999) but rates remain lower than those of in-vivo stimulated cycles, indicating that optimization of IVM remains a challenge (Goud *et al.*, 1998). The primary problem in oocytes matured *in vitro* is reduced developmental competence, particularly cleavage and development beyond the 4-cell stage (Trounson *et al.*, 1994). This was apparent in our study: the most advanced embryo observed reached 6 cells with most embryos arresting around the second cleavage division (3–4 cells). However, it appears that if the initiation of maturation is triggered *in vivo*, then developmental potential increases (Chian *et al.*, 1999) although the value of priming with low-dose FSH remains unclear (Wynn *et al.*, 1998; Mikkelsen *et al.*, 1999). Others have shown the relative effects of follicular versus luteal phase retrieval of oocytes (Cha and Chian, 1998; Whitacre *et al.* 1998), underlining the importance of the endocrine environment. In our study, PCO patients were not given any form of hormonal manipulation prior to oocyte retrieval; however, it is likely that induction of a withdrawal bleed with or without late follicular administration of HCG (Buckett *et al.*, 1999 Chian *et al.*, 1999) may improve the maturation rates achieved in the present study. These methods still have the benefit of avoiding the major element of the drugs normally administered for ovarian stimulation.

Evidence is accumulating that MAS is an important endogenous factor involved in promoting oocyte maturation. The data presented here show positive effects of MAS upon survival and maturation of human immature oocytes collected from unstimulated PCO patients and stimulated ICSI patients respectively. The limitations of a morphological assessment of viability are acknowledged, but the choice of criteria was supported by earlier experiments using vital stains. Clearly the use of an invasive method in this study would have precluded the collection of data on further development. This is an important area since it is not yet known whether embryos resulting from IVM in the presence of MAS have the potential to develop further or implant. In mouse cumulus cells, a heat stable meiosis activating substance is stimulated by FSH (Byskov *et al.*, 1997; Yding Andersen *et al.*, 1999) and artificial elevation of MAS in cumulus cells using inhibitors of specific enzymes on the steroid synthesis pathway promotes maturation of mouse oocytes *in vitro* (Leonardsen *et al.*, 2000). MAS was shown to improve immature oocyte function in mice by supporting microtubule assembly and delaying the release of cortical granules (Hegele-Hartung *et al.*, 1999).

Results were recently presented (Grøndahl *et al.*, 1999 2000) of IVM of 81 human oocytes allocated into several groups, with or without MAS, for histological analysis. These data showed a significant ($P < 0.05$) increase in the proportion of immature oocytes completing maturation after 30 h *in vitro* in the presence of 20 μmol/l MAS; however, at 22 and 40 h the difference was not significant. Grøndahl's study differed from ours in several respects. His patient group had polycystic ovaries, but received oral contraception for 2 months, to which was added a gonadotrophin-releasing hormone (GnRH) agonist for pituitary down-regulation, followed by recombinant FSH for 3 days. Follicles of 8–12 mm were aspirated on days 7–9 and all oocytes were cumulus-enclosed throughout culture. These patients therefore underwent stimulation cycles. Also half their aspirated oocytes were used for infertility treatment although HCG was not administered. The medium used by Grøndahl *et al.* for the research oocytes was similar to ours, being M199 supplemented with 0.29 mmol/l pyruvate antibiotics and 0.8% HSA, with or without MAS which was prepared in EtOH. None of their research oocytes was inseminated.

In this study, we have demonstrated oocyte maturation with developmental competence as far as the second cleavage division. The effects of MAS on embryo development could

not be analysed statistically in view of the low numbers of embryos generated. The lower maturation rate observed in the oocytes originating from unstimulated PCO was probably due to compromised follicle development as a result of the abnormal endocrine environment, which could also account for the higher rate of atresia observed in the GVBD oocytes, compared with the ICSI patient group. In PCO patients, GVBD oocytes were probably retrieved from partially atretic follicles, where granulosa cells have dissociated from the oocyte and factors controlling meiotic arrest have been lost. Oocytes in atretic follicles may often be found to have progressed further than meiotic prophase I (Anderson *et al.*, 1997).

In our study, the effects of MAS alone have been studied, although addition of FSH to the medium might augment MAS production in the cumulus (Byskov *et al.*, 1997) and the inclusion of FSH, HCG and/or growth factors *in vitro* has already been demonstrated to stimulate IVM by others (Durinzi *et al.*, 1997). We chose to omit these other factors so that the effects of MAS alone could be established in the absence of confounding influences. We felt that this was important, even though a higher overall maturation rate might be gained by a combination of supplements in the medium.

In conclusion, oocytes derived from stimulated ICSI patients and unstimulated PCO patients constitute different populations, probably due to their differing endocrine and intrafollicular environments. MAS exerts positive effects upon different aspects of oocyte function in both groups. In view of its role as an intrafollicular steroid, and its ability to promote maturation, its potential for further development as an in-vitro meiotic stimulant should be evaluated.

Acknowledgements

The authors are grateful for assistance from staff in the gynaecology and day case theatres at Walsgrave Hospital who participated in the immature oocyte collections. We also wish to thank staff at the Centre for Reproductive Medicine who assisted with the recruitment of patients and laboratory procedures, particularly Miss M.Mudhar. We are especially grateful to our patients for consenting to participate in the study. We are also grateful to the Mason Medical Research Foundation and the Gift of a Life Appeal for contributing to the purchase of some specialized equipment used in this study. Funding was provided by grants from the West Midlands Regional Health Executive to G.M.H. and L.D.K., the Walsgrave Hosptials R&D Committee to G.M.H. and C.R.K., and from the Danish Medical Research Council, grant no. 9400824 and the Danish Environmental Research Programme, grant no. 9700832 to A.G.B. and M.B.

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Received on August 31, 2000; accepted on November 20, 2000